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HUBERT MAITLAND TURNBULL, D.M., F.R.S.  
ON HIS 70TH BIRTHDAY  
3RD MARCH 1945

WK 1936



*Hubert M. Turnbull*

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## BACTERIOPHAGE ACTION ON *BACT. TYPHOSUM* AND *B. MEGATHERIUM* AS DISPLAYED BY DARK GROUND CINEMICROGRAPHY

ADRIANUS PIJPER

*From the author's private laboratory, Pretoria, South Africa*

(PLATES I-III)

### *Structure of the bacterial cell*

According to Knaysi (1938) a bacterial cell has three "membranes". The outermost is a slime layer, not visible under dark ground illumination. Next there is the cell wall, a thin, tough and rigid sheath which gives the cell its shape. Ordinarily this cell wall is not visible in dark ground because it is in close apposition to the third, very brilliant, "membrane". It is, however, believed to become visible during plasmolysis, and recently it was photographed by Mudd and his co-workers (1941) with an electron microscope after the bacteria had been broken up by a sonic oscillator. The third "membrane" is a layer of condensed cytoplasm, which lines the cell wall. This layer is very brilliant in dark ground, it is much thicker than the cell wall, it varies in thickness with the age and the activities of the cell, and its inner border surrounds the inner and more liquid portion of the cytoplasm. It is this more liquid central cytoplasm which appears dark in dark ground. The dividing line between the brilliant condensed layer and the dark central region is not sharp. It can be made to shift its position with the amount of light used. It seems likely that this layer of condensed cytoplasm is the seat of the main metabolic activities of the cell. Slight changes in its

constitution and outline can be witnessed in normal cells. Normal cell death is sometimes accompanied by similar but deeper changes. It will be shown that bacteriophage activity primarily produces rapid, profound and irreversible changes in this layer.

### *Previous observations on the mode of action of bacteriophage*

Bronfenbrenner (1928 *a* and *b*, 1933) reviewed the early literature and added extensive observations, partly from a film made in collaboration with others (1927), partly based on stained preparations and on ultraviolet microscopy. He concluded that a bacteriophage excites metabolic activities and that lysis is a secondary phenomenon caused by osmotic changes in the cell. Bayne-Jones and Sandholzer (1933), in cinematographic studies of phage action, saw swelling and explosive lysis in *Bact. coli*, whilst *B. megatherium* just disappeared slowly. They consider reduction in surface tension as a possible cause of lysis. Wollman (1925) had seen dysentery bacilli swell, but no explosions took place, and *Bact. coli* just became granular. d'Hérelle (1921), who made observations by dark ground illumination, described very fine granules entering the bacterial cell, causing it to swell and finally burst, with the dispersal of the very fine granules which he believed to be his ultra-microbes. Merling-Eisenberg's magnificent photo-micrographs (1938) show *Bact. coli* filled with granules and then exploding, releasing large numbers of small uniform bodies giving a blue diffraction colour, which "blue bodies" he takes to be bacteriophage particles.

### *Technique*

My own observations were made with the sunlight dark ground technique previously described (1930, 1931-32, 1938, 1940, 1941 *a* and *b*, 1942). Sunlight, being more powerful than any other source of light, brings out many otherwise hidden details. It also allows the making of motion pictures of the happenings, a method of study particularly suitable for this subject. Running the film backwards is particularly instructive and is a unique method of research, because the disappearance of a bacterium due to the action of phage can be traced backwards through all its phases. Several thousand feet of 16 mm. cinema film were exposed. Selected parts were finally put together and form a continuous record of the various ways in which bacteriophage affects bacteria. Isolated pictures from the film are used to illustrate this paper.

The filming method of investigating microbic life has additional advantages. It disciplines observation. One can only film clear-cut happenings. Disturbing factors must be completely eliminated so as not to spoil the picture. Also the camera often records what the eye may miss, though it must be conceded that even with present-day film not everything that the eye can see can as yet be photographed.

I have used Kodak Super XX film throughout. The magnification on the film was usually  $\times 320$ . The Movikon camera ran at 12 pictures per second, giving an exposure time of  $1/25$ th second. Zettnow's liquid green filter in appropriate dilutions was sometimes used to reduce glare. The lenses mostly used were a Zeiss oil-immersion  $\times 60$  with iris diaphragm in combination with a Zeiss compensating eyepiece  $\times 10$ , and a Beck oil-immersion  $\times 90$  with funnel in combination with a Zeiss compensating eyepiece  $\times 7$ . The dark ground condenser was Siedentopf's cardioid. The camera was used without its own lens. A "beam splitter", i.e. a device which enables one to view the microscopic field whilst it is being filmed, is of course essential.

*Material used*

Observations were made on the Bhatnagar strain of non-motile typhoid bacilli Vi I and on a local strain of *B. megatherium* which had lost its motility. The respective bacteriophages came from sewage and were repeatedly isolated from plaques. Both cleared broth suspensions in a few hours at room temperature.

A drop of filtered lysed broth culture, mixed on the usual mica glass microscope slide with a small loopful of a young culture and then covered with a large coverglass, provided a suitably thin preparation in which it was possible to get only one or two bacilli in a field. The bacteriophage filtrates were sometimes diluted to slow down their action. Brownian movement often interfered with prolonged observation of a particular bacillus, but it also happened that a bacillus got anchored to the slide or coverslip and was kept in focus quite easily.

*Normal bacilli in dark ground*

Normal bacilli in sunlight dark ground appear as rod-shaped structures with a very brilliant outline of varying thickness (fig. 1). The apparent thickness can be made to vary with the brightness of the light used. The central area, however, is always dark and in normal bacilli I have never seen any particles in this more liquid portion of the cytoplasm. The periphery of the bacillus shows a smooth sharp outline, representing the outer border of the cell wall. The brilliant layer of condensed cytoplasm lining the cell wall is all that shows up in a normal bacillus in dark ground and gives the bacillus its typical appearance.

*Action of bacteriophage: initial changes*

Whatever the final outcome of bacteriophage action, the initial changes in the bacilli are always the same. A recently filtered suspension of bacteriophage in sunlight dark ground shows a very large number of particles, many of which appear to be of uniform size as indicated by their definite blue diffraction colour (fig. 2). These are the particles described by Merling-Eisenberg as "blue bodies". That ordinary broth exhibits similar though less numerous particles is no argument against the assumption that these "blue bodies" in a filtered phage suspension are or contain phage bodies. In a mixture of bacteriophage and normal bacilli these bodies often impinge upon the bacilli, giving a definite impression of attack. It is not possible to see whether they penetrate or not: at any rate they are not repelled and they seem to disappear when they come close to the bacillus. Burnet recognised similar bodies and had them photographed by Barnard before and after agglutination (1933a); he assumed that they were adsorbed by the bacteria (1933b). In fig. 3 there are three "blue bodies" approaching the bacillus.

The first change in the bacilli as a result of bacteriophage activity was a loss of brilliancy of the layer of condensed cytoplasm. This proved useful as a guide. The fate of bacilli which had lost their



brilliancy usually proved worth following up, as by no means all bacilli undergo changes at the same time or rate. Fig. 4 shows typhoid bacilli exhibiting this dull appearance of the layer of condensed cytoplasm. In the film this is seen to be followed by the changes shown in figs. 5-8. Similar initial changes resulting from bacteriophage action on *B. megatherium* are shown in figs. 16-22. These initial changes resemble somewhat the appearance of some of the normal bacteria of fig. 1, with the important difference that when they occur as the result of bacteriophage action they are irreversible and progressive. The layer of condensed cytoplasm no longer forms a smooth continuous lining of the cell wall but begins to collect in smaller or larger lumps lying along it. The poles of the cell are favourite places for larger aggregations of condensed cytoplasm, which often again exhibit great brilliancy.

These alterations in the appearance of the layer of condensed cytoplasm are the initial changes which all bacilli successfully attacked exhibit in some degree. They are the forerunners of further changes of various kinds, some of which come on quite quickly while others take long to develop.

#### *Action of bacteriophage: later stages*

Once the layer of condensed cytoplasm has undergone these initial disintegrative changes, the destructive process can take one of several courses. A common course for both typhoid bacilli and *B. megatherium* is a further continuous but slow disintegration of the layer of condensed cytoplasm. For typhoid bacilli this is illustrated by figs. 9-15. Its total mass breaks up into a number of lumps and particles distributed irregularly over the bacillus. The cell keeps its shape owing to the presence of the cell wall, which now becomes apparent as a thin line surrounding the bacillus, and lacking the brilliancy of the bits and pieces of the layer of condensed cytoplasm. The remains of this layer seem to adhere to the wall, although in some cases and at some stages one may gain the impression that small particles of condensed cytoplasm are lying in the more liquid central region. The whole picture may have given d'Hérelle the impression that the bacillary body had become filled with bacteriophage particles. It probably corresponds to what Bronfenbrenner has called the "beaded appearance" of disintegrating bacilli. The film shows clearly that it is the disintegrating layer of condensed cytoplasm which gives rise to pictures like figs. 14 and 15.

In the case of *B. megatherium*, figs. 16-28 tell a similar story. Here too the layer of condensed cytoplasm gradually becomes a collection of particles distributed in irregular fashion along the cell wall, which stands out again as a matt outline of the bacillus and obviously consists of different material. It seems likely that the free spaces on the wall between the particles of condensed cytoplasm are

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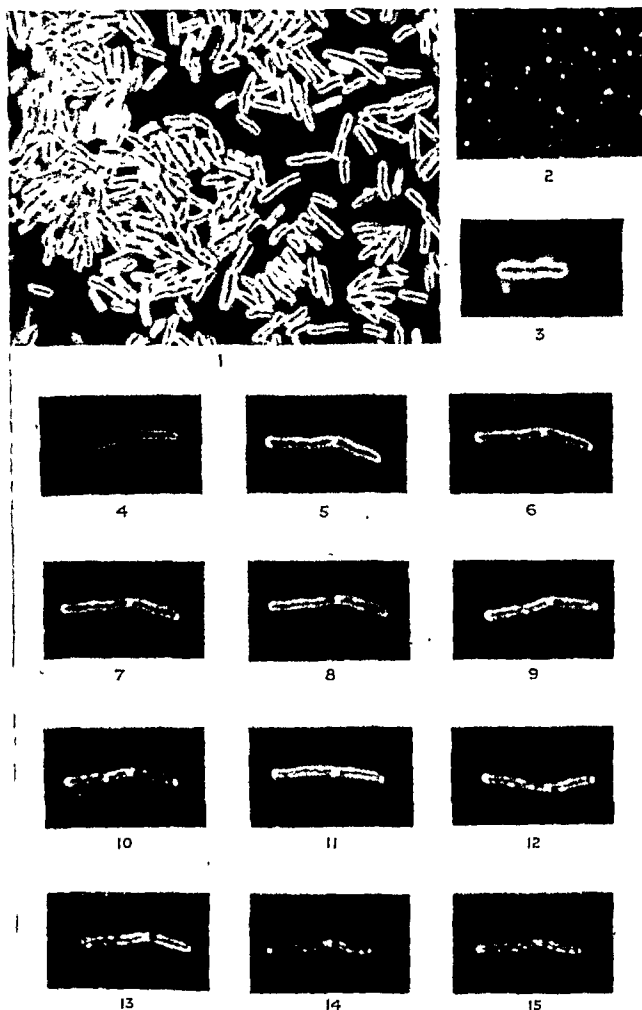


FIG. 1.—Normal typhoid bacilli.  $\times 2500$ .

FIG. 2.—Mostly "blue bodies" in suspension of bacteriophage.  $\times 1600$ .

FIG. 3.—Typhoid bacillus with "blue bodies" attached or becoming attached.  $\times 2500$ .

FIGS. 4-15.—Gradual disintegration of layer of condensed cytoplasm in two typhoid bacilli. Note emergence of the continuous cell wall.  $\times 2500$ .



responsible for d'Hérelle's impression that there were holes in the wall through which bacteriophage particles could escape.

In many cases the fragments of the layer of condensed cytoplasm do not lie still inside the cell wall: the film shows instances where they are in violent motion, so much so that the whole bacillus vibrates. In the case of the two bacilli of figs. 34 and 35, after the transition from fig. 34 to fig. 35 had taken place, one of them vibrated so much that it shook itself loose from the other.

Once a bacillus has reached the more advanced stages of disintegration of its layer of condensed cytoplasm, there is no turning back, and such a bacillus may be regarded as dead. It is not conceivable that it could perform its normal functions after such thorough disintegration of its cytoplasm. The cases therefore where bacteriophage action takes the course of slow disintegration of the layer of condensed cytoplasm provide microscopic confirmation that death and lysis in those circumstances are separate processes. This was already known from the work of Andrewes and Elford (1932), who found that by adding sodium citrate, which inhibited multiplication of their phage, to a mixture of *Bact. coli* and excess of phage, the bacilli were first killed and lysis followed many hours later.

In the case of *B. megatherium* I have always seen the sequence as depicted in figs. 16-28. After the slow disintegration of the layer of condensed cytoplasm, the further disintegration of the remains of the bacillus always followed the course illustrated by figs. 29-33. For a long time pieces of cell wall, showing up as thin matt lines to which brilliant particles of the condensed cytoplasm are attached, remain visible. Small particles resembling "blue bodies" keep coming off and gradually the mass of debris is dispersed into the surrounding fluid.

The effect then of a bacteriophage on *B. megatherium* is rather simple and stereotyped. In the case of *Bact. typhosum* the events are often more spectacular. A typhoid bacillus which has reached the state shown in fig. 15 usually undergoes the same slow dispersal as is customary with *B. megatherium*. But not all typhoid bacilli reach this high degree of cytoplasmic disintegration by way of the gradual process mentioned so far. Some begin by going slightly "matt" in their layer of condensed cytoplasm, and then, after slight alterations in the configuration of this layer, it quite suddenly disintegrates. The bacillus in fig. 36 reached the state shown in fig. 37 with a sudden jump, in a fraction of a second. This dramatic event I have filmed on several occasions. Complete dissolution of the whole bacillus may then follow the ordinary slow course. However, when a state of high disintegration of the layer of condensed cytoplasm has been reached by a typhoid bacillus, whether gradually or suddenly, it does not always just fade away like *B. megatherium*. It may gradually shrink, as shown in figs. 38-41, where a bacillus rolled itself up into a ball from which small particles kept flying off. The opposite of this

—increased internal tension—is illustrated by one of the bacilli in fig. 42, which, after the usual disintegration of its layer of condensed cytoplasm, suddenly blew out its thin wall into a terminal bubble (fig. 43), followed by further bubbles along the wall. In such cases the separate existence and toughness of the cell wall become particularly obvious, and the usual end result is gradual total dispersal. Yet the production of bubbles from the cell wall does not always take place in this deliberate and localised fashion. The disorderly mass of bubbles with fragments of condensed cytoplasm attached, which is shown in fig. 45, was the fairly well organised bacillus of fig. 44 only a fraction of a second before. The bacillus simply blew itself up. In such a case the remains, consisting of thin-walled bubbles—evidently parts of the old cell wall with the brilliant particles of condensed cytoplasm attached—may hang together for quite a time before they disappear completely. All this time small particles like “blue bodies” escape from the disorderly mass into the surrounding fluid. In other cases again, as in fig. 46, after minor alterations in the appearance of the layer of condensed cytoplasm just visible in the photograph, within a 25th of a second the lower bacillus had blown itself to pieces, leaving nothing but barely visible thin-walled bubbles (fig. 47). Finally a typhoid bacillus may, after the usual initial changes shown in fig. 48, suddenly disrupt into a mass of small bodies (fig. 49), many of which look like “blue bodies”, and with numbers of apparent “blue bodies” coming off for a long time, without showing any remnants of cell wall.

### Summary

The changes caused in *Bact. typhosum* and *B. megatherium* by bacteriophage action were studied by means of sunlight dark ground microscopy. A 16 mm. cinemicrographic film made at the same time proved useful in the further study of the happenings, especially for tracing back the history of affected bacilli.

Bacteriophage suspensions showed numbers of small particles with a blue diffraction colour, hence called “blue bodies” as suggested by Merling-Eisenberg. These were seen to get into close contact with the bacilli and, during or after the various processes of disintegration which the bacilli underwent, numerous “blue bodies” were seen to come out of the remains.

The first effect of bacteriophage action was always a change in the layer of condensed cytoplasm which forms the third “membrane” of the bacillus. This layer first lost its brilliancy and then broke up into particles and larger aggregates which remained attached to the cell wall at irregular intervals, exposing the cell wall to view in the intervening spaces. This disintegration of the vital layer of condensed cytoplasm may be taken to mean the death of the cell. In *B. megatherium* the process of disintegration of cytoplasm was

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FIGS. 16-33.—Gradual disintegration of layer of condensed cytoplasm in *B. megatherium*, followed by gradual disintegration of whole bacillus. Note cell wall. Fig. 27 is over-printed.  $\times 1900$ .







### PLATE III

FIGS. 34 and 35.—Gradual disintegration of layer of condensed cytoplasm in two typhoid bacilli. Slightly over-exposed.  $\times 2500$ .

FIGS. 36 and 37.—Sudden disintegration of layer of condensed cytoplasm in a typhoid bacillus. Note cell wall.  $\times 2500$ .

FIGS. 38-41.—Gradual disintegration of layer of condensed cytoplasm in a typhoid bacillus, followed by shrinking of the body.  $\times 2500$ .

FIGS. 42 and 43.—Gradual disintegration of the layer of condensed cytoplasm in *Bact. typhosum*, followed by expansion of the cell wall into a "bubble".  $\times 2500$ .

FIGS. 44 and 45.—Sudden transition of a typhoid bacillus with damaged layer of condensed cytoplasm into a mass of "bubbles", consisting of cell wall with fragments of cytoplasm attached.  $\times 2500$ .

FIGS. 46 and 47.—Sudden disappearance of lower typhoid bacillus, leaving barely visible remnants of the cell wall.  $\times 2500$ .

FIGS. 48 and 49.—Sudden change of a typhoid bacillus into a mass of fragments of condensed cytoplasm and "blue bodies".  $\times 2500$ .

ACTION OF BACTERIOPHAGE ON BACILLI



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always slow, in *Bact. typhosum* it sometimes happened quite suddenly. *B. megatherium* after this disintegration always disappeared in the same way—by slow dispersal of the cell. *Bact. typhosum*, after reaching the stage of internal disintegration, whether gradually or suddenly, could also disappear by slow dispersal, but often exhibited more spectacular conduct. Sometimes it shrank into a small mass, sometimes blew bubbles from the thin cell wall, sometimes exploded and became a mass of bubbles with particles of cytoplasm attached, or just a mass of bubbles or of small particles. These processes were usually accompanied by the release of numerous "blue bodies".

It appears therefore that in the cases investigated the bacteriophage acts by destroying the vital layer of condensed cytoplasm, thus killing the cell. What follows depends on activities and forces within the cell which vary in different kinds of bacteria and even in different individuals of the same strain.

Part of the apparatus used in this investigation was acquired through a grant from the Research Grant Board of the Union of South Africa.

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# TISSUE CHANGES IN EXPERIMENTAL MICE TREATED WITH PENTOSE NUCLEOTIDES

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(PLATES IV-IX)

CELLULAR changes of a systemic nature occurring in the tissues of experimental mice have already been described (Parsons, 1935, 1936, 1938, 1942, 1943). Mice bearing primary or grafted sarcomas show progressive changes in the blood, lymphoid tissue, spleen and liver, which are initiated by and associated with the development and growth of the tumours. A leucocytosis of a myeloid type and often of leukæmic proportions is a common feature. Reticulosis, plasmacytosis and myeloid metaplasia in lymph nodes are also present. Mice bearing grafted generations of sarcomas induced by methylcholanthrene (Parsons, 1942) show amyloid infiltration of the spleen, the incidence of this condition being as high as 93 per cent. in the pure line (CBA) mice in which the primary tumours occurred. Similar amyloid changes also affect the liver in many of the tumour-bearing mice, both irradiated and non-irradiated (figs. 7, 8 and 14). Amyloid infiltration of the spleen and liver has not been noted in normal mice, in those subjected to general irradiation, or (before tumour development) in those undergoing treatment with the Mal. compound or methylcholanthrene.

Since the blood and lymphoid changes in these tumour-bearing mice suggested increased activity of the fixed reticulum cells of the hæmopoietic tissues consequent on the introduction and growth of the sarcomas, it was felt to be of interest to test the effects of injections of a compound known to act on such primitive undifferentiated cells. For this purpose a commercial mixture of pentose nucleotides used clinically in cases of acute agranulocytosis was employed. It was found that changes corresponding with singular exactness to those noted in the tissues of tumour-bearing mice were induced. The results are set out below.

## *Groups of mice treated with pentnucleotides*

1. Normal mice (17 stock mice).
2. Generally irradiated mice (6 stock mice).
3. Mice treated with silica (5 stock mice).
4. Mice treated with carcinogenic agents (24 stock mice).
5. Mice bearing grafted generations of sarcomas induced by methylcholanthrene (65 pure line (CBA) mice and 3 stock mice).

*Pentnucleotides.* The mixture of pentose nucleotides supplied commercially by Messrs Menley and James was used; this is stated to contain the sodium salts of the four nucleotides of ribonucleic acid of yeast supplied in sterile aqueous solution of pH 7.2. The solution contains approximately 8 per cent. of the salts preserved with 0.3 per cent. cresol. The terms nucleotide and pentnucleotide in this paper refer in all cases to this mixture and not to the individual nucleotides.

*General irradiation and induction of tumours by carcinogenic agents.* These have already been described (Parsons, 1942). X-radiated mice received a single dose of 600r. previous to treatment with pentnucleotide. Mice treated with silica received a single massive dose by means of a trochar and cannula into the right flank before injection of the mixture and, together with mice of the other groups, were injected 5 times a week in the left flank with an average dose of 0.5 c.c., equivalent to 0.035 g. pentnucleotides. This treatment was continued for 3-6 months in groups 1-4.

*Staining methods.* The Unna-Pappenheim method was used for demonstrating plasma cells and Robb-Smith's modification of Foot's method for reticulin. Spleens and livers showing amyloid infiltration were stained with Congo red and methyl-violet 5B (see Addendum by Professor Turnbull). Weigert's method was used for fibrin.

*Data for normal mouse blood and splenic weights.* The white blood cell count ranges from 7000 to 20,000 per c.mm.; 21,000-40,000 constitutes a moderate leucocytosis and counts above this are considered to be of leukæmic type. The average splenic weight of young normal mice has been found to be 0.16 g. (range 0.118-0.217 g.). In old mice the splenic weight is considerably higher, ranging from 0.119 to 0.482 g. (average 0.322 g.). Fig. 6 shows the microscopic appearance of a normal mouse spleen.

## EXPERIMENTAL RESULTS

### *Normal mice treated with pentnucleotide*

Prolonged dosage over 4-6 months has not been found to induce tumour formation, but marked changes occur in the tissues similar to those noted in mice bearing primary or grafted sarcomas. A leucocytosis of a swinging type is present and may rise as high as 62,000. Absolute values of both lymphocytes and polymorphs are increased, as shown in fig. 1. In each case the first injection of pentnucleotide was given on the day following the normal count. Comparison of the blood counts of these mice with those of normal control mice injected with normal saline (counts 3 and 4 in fig. 4) shows that the total count in the latter is almost invariably contained within the normal range, while the polymorph level is consistently low. In mice injected with nucleotides the polymorph count is both higher and fluctuating, and it may cross the lymphocyte count in a similar manner to the crossing observed in counts from tumour-bearing mice. Count 3 in fig. 1 is very like counts 1 and 2 in fig. 4, taken from mice grafted with carcinoma 63. Occasional very large cells (probably hæmocyto blasts) and mitotic figures are met with in blood films and the percentage of immature white cells is increased.

The lymph nodes may be normal or increased in size. They show great activity of reticulum cells, especially at the periphery. The littoral cells run down in broad strands into the medulla and a brightly staining groundwork of such cells is noticeable through the gland. These cells are associated with an increase of plasmacytoid cells, which often show mitoses and twin nuclei (fig. 18). Myeloid cells are frequent (figs. 16 and 17) and nuclear pyknosis of lymphocytes in the germinal centres sometimes occurs. Giant cells are found in some cases (fig. 16). Detached littoral cells in the sinuses frequently appear to be full of red blood cells and large cells full of highly

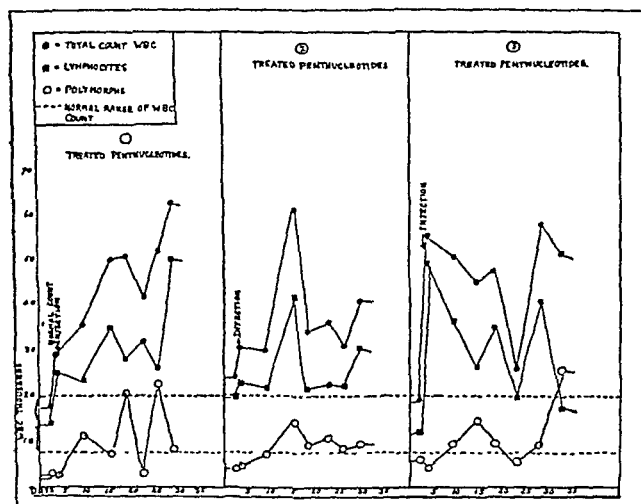


FIG. 1.—Representative blood counts from normal mice injected with pentnucleotides.

refractile droplets may also occur. The prussian blue reaction was not investigated.

Amyloid infiltration of the spleen similar to that noted in mice bearing grafted generations of methylcholanthrene sarcomas has been found in a considerable number of the mice treated with nucleotides. The infiltration appears to start as hæmorrhagic areas round the Malpighian bodies, which become atrophied. Homogeneous patches staining brightly with eosin develop among the red blood cells, these areas finally coalescing in circles about the remaining lymphoid tissue and giving the characteristic reactions for amyloid (figs 7 and 9). Weigert's method for fibrin gave negative results.

Amyloid infiltration of the liver was also noted in mice injected



with pentnucleotides, resembling that occurring in the liver of mice grafted with methylcholanthrene sarcomas (figs. 14 and 15). Round-cell infiltration about the portal tracts may be massive and activity of Kupffer cells is apparent.

In this group the size of the spleen, especially in old mice, tended to be diminished, the splenic weight ranging from 0.102 to 0.254 g.

### *X-radiated mice and mice treated with silica*

In neither of these groups was there any indication of tumour formation after several months' treatment with nucleotides. Blood

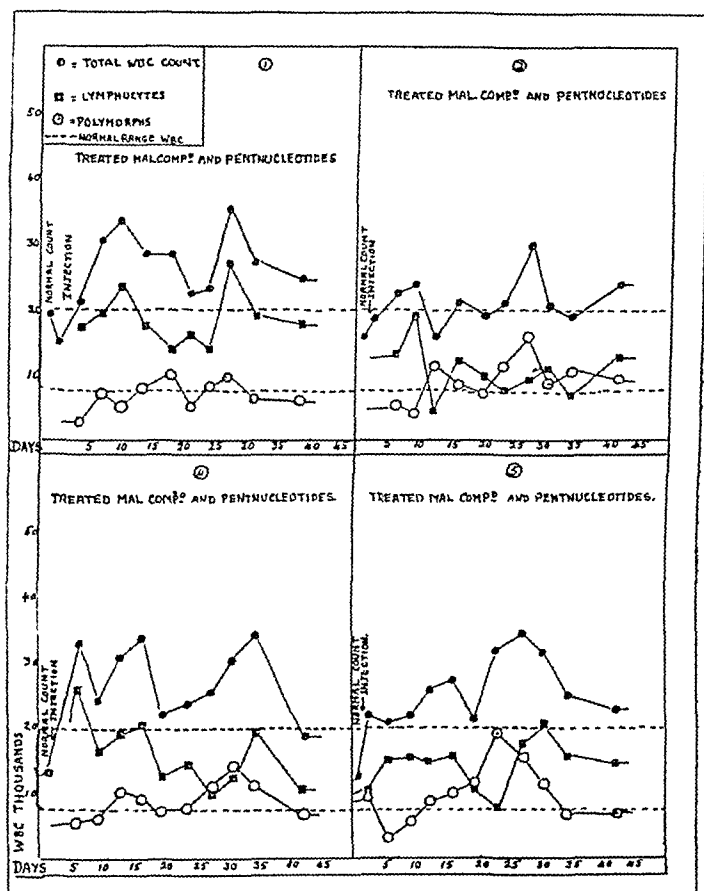


FIG. 2.—Representative blood counts from normal mice injected with the Mal. compound and pentnucleotides and before tumour development.

counts were not made. Changes in the lymph nodes and amyloid infiltration of the spleen were similar to those found in both normal treated and tumour-bearing mice (figs. 8-11, 17 and 18). The size of the spleen was not diminished, the average weight for X-radiated mice being 0.297 g. (range 0.275-0.363 g.) and for normal



#### PLATE IV

FIG. 6.—Section of spleen of normal mouse. H. and E.  $\times 95$ .

FIG. 7.—Macroscopic appearance of spleen from (a) mouse bearing grafted generation of methylcholanthrene sarcoma 1, (b) mouse injected with pentnucleotides showing similar areas of amyloid infiltration.  $\times 4$ .

FIG. 8.—Spleen of mouse bearing 25th grafted generation of methylcholanthrene sarcoma 2, showing areas of amyloid infiltration among the lymphoid tissue. H. and E.  $\times 95$ .

## TISSUE CHANGES INDUCED BY PENTOSE NUCLEOTIDES



FIG. 6

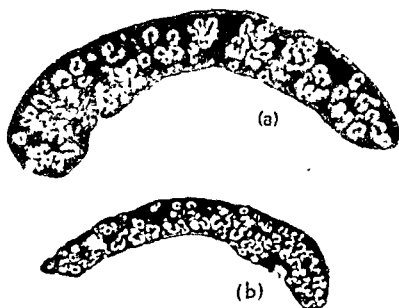


FIG. 7





silica-treated mice 0.335 g. (range 0.291-0.422). In normal mice subjected to general irradiation the size and weight of the spleen have usually been much diminished. If however an X-irradiated mouse is grafted either subcutaneously or intraperitoneally with a sarcoma, the size and weight of the spleen have usually been increased. In this respect the action of pentnucleotide is similar to the effect of a grafted tumour. No explanation can be offered for this reaction. Amyloid infiltration of the liver was not observed.

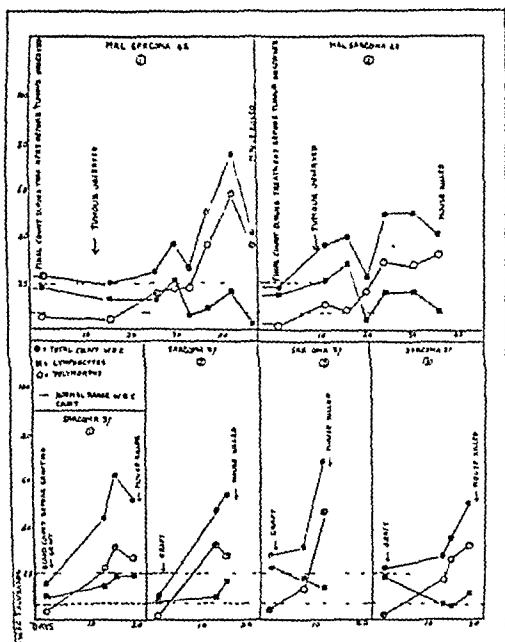


FIG. 3—Blood counts from (a) mice bearing primary Mal sarcomas and (b) grafted generations of sarcoma 37, showing rise in total count and polymorph count at the time of tumour development and the characteristic crossing of the lymphocyte count by the polymorph.

#### *Mice treated with carcinogenic agents*

Injection of nucleotides did not appear to accelerate tumour formation in mice treated with the Mal. compound, no sarcomas having developed in the space of 4-6 months. In irradiated and non-irradiated mice injected with the Mal. compound alone it has been found that blood counts are maintained almost invariably within

the normal range, and that the polymorph-lymphocyte ratio is unchanged up to the time of tumour formation. When a sarcoma develops, the total count is rapidly increased and the polymorph-lymphocyte ratio is reversed (figs. 3 and 5). Comparison of figs. 2 and 5 shows that mice injected with both pentnucleotide and the Mal. compound develop blood counts of a different type, the total count showing a moderate leucocytosis, with increase in the absolute

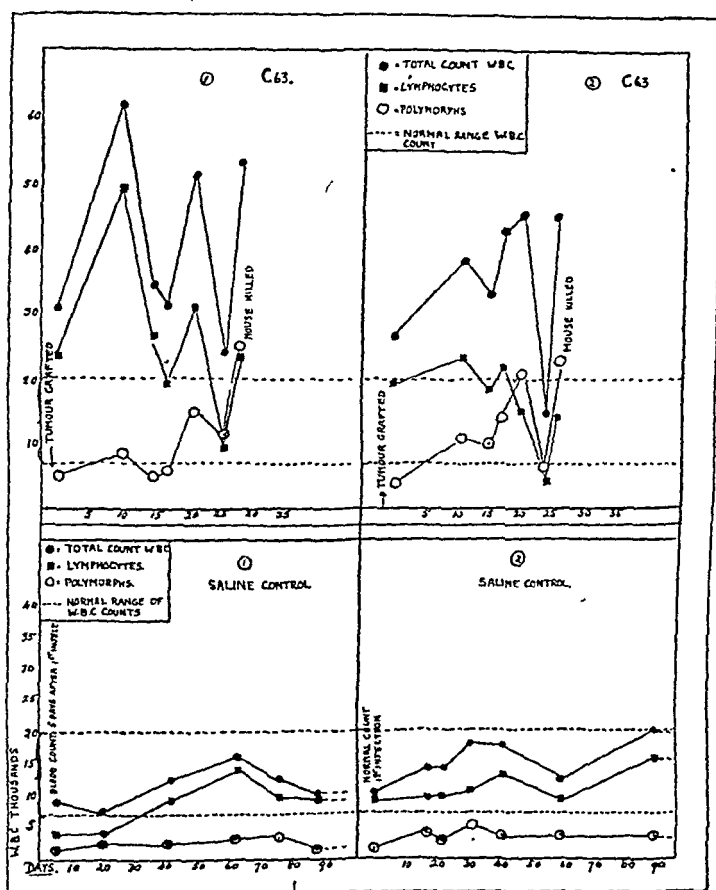


FIG. 4.—Blood counts from (a) mice bearing grafted generations of carcinoma 63 and (b) normal control mice injected with saline.

values of the polymorphs. Crossing of the lymphocyte by the polymorph count is common, and immature white cells and haemocytoblasts are found in films.

Lymphoid and splenic changes are similar to those noted in preceding groups (fig. 12). Splenic weights fall within the normal range (0.135-0.283 g., average 0.2 g.).

Mice treated with methylcholanthrene and nucleotides developed primary tumours during treatment and only one mouse killed before

## TISSUE CHANGES INDUCED BY PENTOSE NUCLEOTIDES

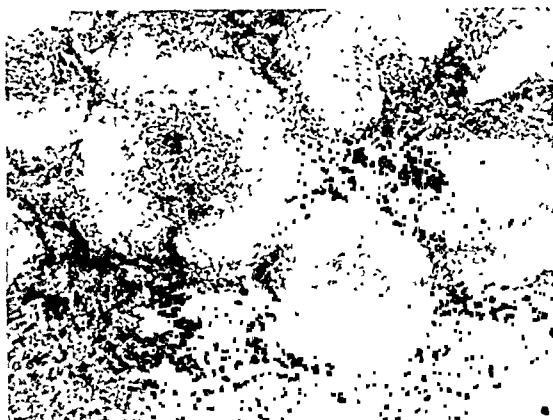


FIG. 9.—Spleen of normal mouse injected with pentnucleotides, showing areas of amyloid infiltration similar to those in fig. 8. H and E.  $\times 95$ .

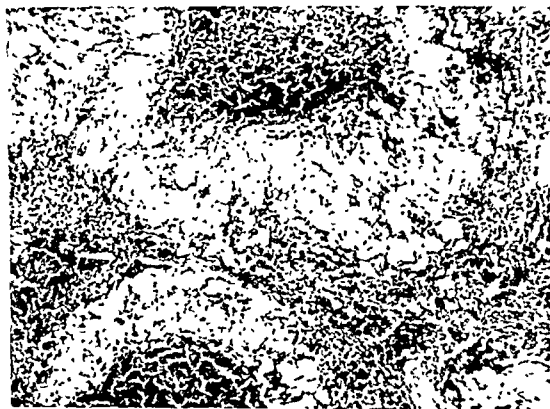


FIG. 10.—Spleen of X-radiated mouse treated with pentnucleotides, showing areas similar to those in figs 8 and 9. H. and E.  $\times 95$ .





tumour formation and showing the action of pentnucleotide is included in this group. A blood count made when this animal was killed showed a leucocytosis of 52,000, with an absolute increase in polymorphs. The lymph nodes showed changes similar to those in the previous groups and early amyloid infiltration of the spleen had occurred (fig. 13).

In 267 stock and pure line (CBA) mice previously treated with the Mal. compound or methylcholanthrene only and killed during treatment or at the time of primary tumour formation, no amyloid infiltration of the tissues was found.

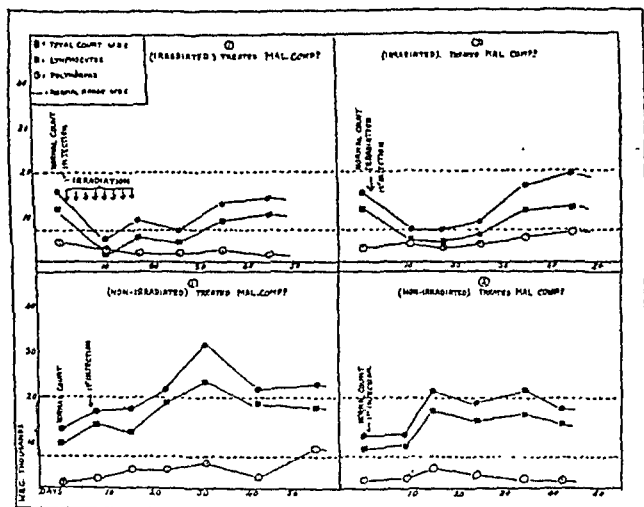


FIG. 5.—Blood counts from irradiated and non-irradiated mice injected with the Mal. compound and before tumour development.

#### *Mice bearing grafted generations of methylcholanthrene sarcomas*

Tissue changes in these grafted mice have already been described (Parsons, 1942, 1943). Numerous tumour-bearing mice were injected with pentnucleotide from the time of grafting, the majority (65 out of 68) being pure line (CBA) mice bearing methylcholanthrene sarcoma 1. It was observed that this treatment frequently increased the size and weight of the tumours as compared with the grafted controls. Old CBA mice appear peculiarly resistant to the growth of the sarcoma, but in 10 old CBA mice grafted with the tumour and treated with pentnucleotide 8 large sarcomas developed. Ten

old control mice gave negative results. Five of the latter were regrafted and injected with the compound: two developed sarcomas later. It was noted that there was a time lag in the formation of the tumours in the old mice as compared with young grafted CBA mice.

Splenic enlargement and amyloid infiltration appear most marked in mice bearing large and rapidly growing tumours. Apparently contradictory results were obtained in the grafted mice treated with pentnucleotide. In a group of 26 of these, although the sarcomas were of large size and rapid growth, the spleens were of normal size and 16 showed no indication of amyloid infiltration. The 95 grafted mice serving as controls showed large spleens, amyloid infiltration being marked in 81. The incidence of this change therefore appeared to have fallen from 85.3 per cent. in the untreated to 38.5 per cent. in the treated. Splenic weights were not taken. It should be noted that amyloid infiltration does not seem to occur where marked myeloid metaplasia of spleen and lymph nodes is present.

Since the tissue changes in normal mice treated with pentnucleotide are so similar to those produced by sarcoma growth it was difficult to judge to what degree the systemic changes in this group were due to the injection of nucleotides or to the growth of the grafted tumour.

The high leucocytosis usually present in the blood of the grafted mice did not seem to be affected by treatment with nucleotides.

### DISCUSSION

From the above observations it will be seen that a close similarity exists in the tissue changes noted in mice treated with nucleotides to those induced by the introduction and growth of a reticulo-sarcoma, namely a leucocytosis of moderate or leukæmic proportions, with relative excess of polymorphs; reticulosis, plasmacytosis and myeloid changes in the lymphoid tissue; the occurrence of giant cells in the lymph nodes; amyloid infiltration of the liver and spleen.

Amyloid infiltration appears to occur very early in non-tumour-bearing mice treated with pentnucleotides, indications of its occurrence being found as early as 17 days from the start of the experiment. Marked infiltration of both liver and spleen was found in a mouse killed on the 29th day after 10 doses of pentnucleotide. The length of time for marked amyloid change to occur, however, varies considerably, the longest period noted being 100 days. Of the stock mice included in groups 1-4, 17 or 33.3 per cent. showed marked amyloid infiltration at the various times of killing, an incidence closely approximating to that obtained in group 5 of tumour-bearing pure line (CBA) mice treated with the compound. Very early amyloid change was noted in the spleen in the majority of the remaining 34 mice of groups 1-4. Wasting of the tissues was observed in all the mice showing marked amyloid infiltration of liver and spleen.

## TISSUE CHANGES INDUCED BY PENTOSE NUCLEOTIDES

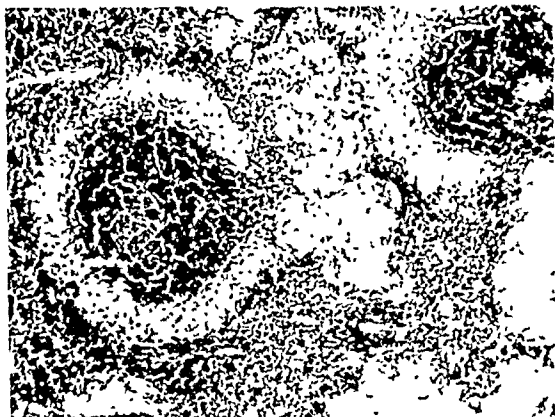


FIG. 11—Spleen of mouse treated with silica and injected with pentnucleotides, showing amyloid condition similar to figs. 8, 10, 11 and 12.  $\times 95$



FIG. 12—Spleen of mouse under treatment with the Mal. compound and injected with pentnucleotides, showing areas of amyloid infiltration similar to figs. 8, 11, H and E.  $\times 95$



## TISSUE CHANGES INDUCED BY PENTOSE NUCLEOTIDES

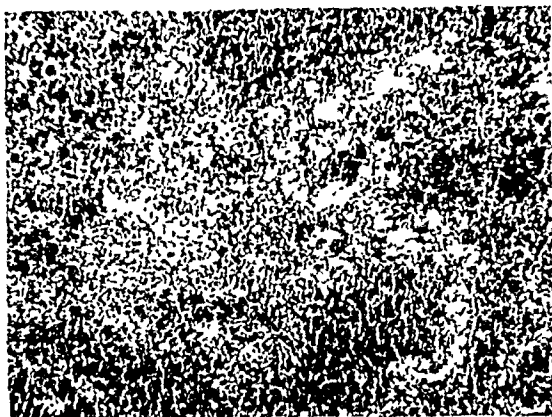


FIG. 13.—Spleen of mouse treated with methylcholanthrene and pentnucleotides, showing early amyloid infiltration. H and E  $\times 105$ .

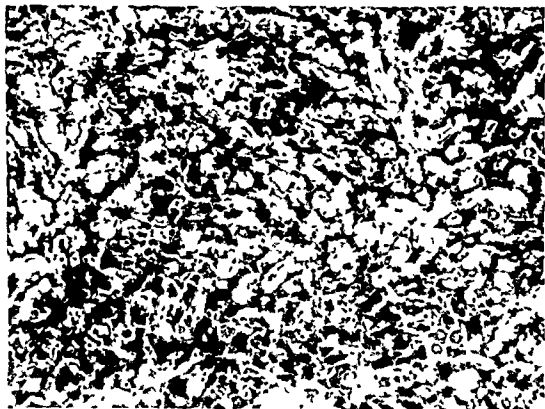


FIG. 14.—Liver of mouse bearing 19th grafted generation of methylcholanthrene sarcoma 2, showing extensive amyloid infiltration with atrophy of liver cells. H and E  $\times 105$ .



## TISSUE CHANGES INDUCED BY PENTOSE NUCLEOTIDES



FIG. 15—Liver of normal mouse treated with pentnucleotides, showing marked amyloid infiltration and great atrophy of liver cells. H and E  $\times 105$

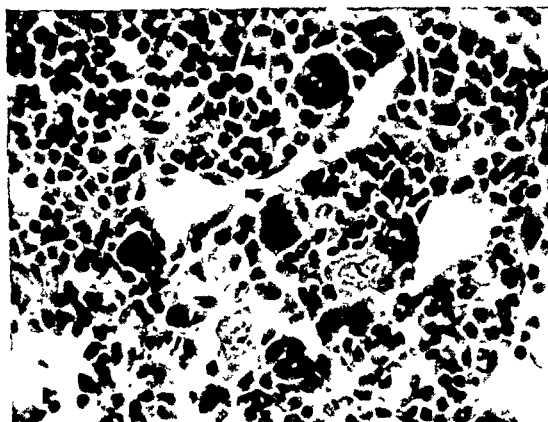


FIG. 16—Mesenteric gland of normal mouse treated with pentnucleotides, showing giant cells developing among lymphoid and myeloid elements. H and E  $\times 640$ .





## TISSUE CHANGES INDUCED BY PENTOSE NUCLEOTIDES

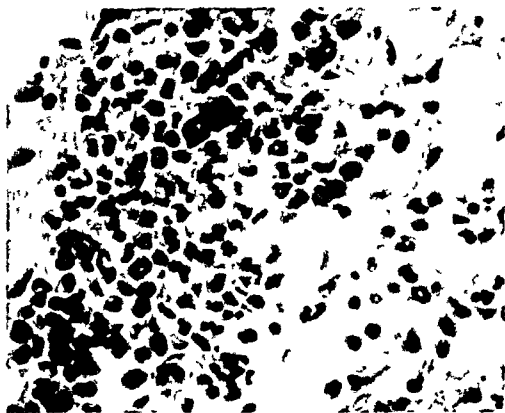


FIG. 17—Mesenteric gland of normal mouse treated with pentnucleotides and silica, showing early myeloid ring forms developing in lymphoid tissue. Note branching reticulum cells forming groundwork of lymph node. H and E  $\times 640$

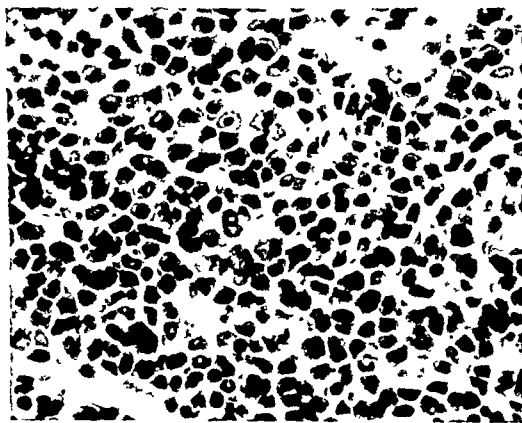


FIG. 18—Section of lymph node of normal mouse treated with silica and pentnucleotides, showing massive plasmacytoid infiltration. H and E  $\times 640$



Since the growth of a sarcoma also induces these systemic effects it has been suggested (Parsons, 1938) that a substance or substances elaborated in tumour cells and liberated into the blood stream may be the causative agent. Should there be a markedly increased content of nucleic acid in the rapidly dividing tumour cells it seems possible that an excess of nucleotides might occur, which, when set free in the blood, could profoundly affect the hæmopoietic tissues and cause changes in the liver and spleen similar to those mentioned.

The importance of nucleotides in relation to embryonic development, repair of damaged tissue and cell division has been recognised in recent years. Gulland (1944) in his Tilden Lecture enumerates the advances made in our knowledge of the essential part played by these compounds in biological processes. Among other noteworthy facts recorded the following are suggestive: (a) there are indications that the increased rate of cell division in tumours is related to changes in nucleic acid synthesis; (b) pentose nucleotides are produced in response to cell injury; (c) nucleic acid plays an essential part in embryonic development; (d) increased ultra-violet absorption by cell cytoplasm after X- and Y-radiation is due to an accumulation of pentose nucleotides; (e) viruses have been consistently found to be nucleoproteins with a definite relationship to the chromosomes, and the active tumour-producing fraction isolated from extracts of the Rous chicken sarcoma is a pentose-containing nucleoprotein associated with a phospho-lipoid component.

It seems possible that such facts may ultimately explain certain of the observations already described. The acceleration of tumour production, both in time and number, in X-radiated mice treated with a carcinogenic compound (Mayneord and Parsons, 1937) may be due to a pre-cancerous condition induced in the irradiated animals by the increase of pentose nucleotides in the cytoplasm. Carcinogenic compounds alone may have a similar action and, by causing cell damage, may evoke a response in the form of increased production of pentose nucleotides, thereby creating an environment suitable for tumour formation. After a more or less prolonged latent period malignant change takes place in the affected tissues. The chemical compounds may not be the actual precipitating agents in the sudden change to neoplastic growth, since cell-free filtrates of extracts of mouse sarcoma can induce tumour growth within 17 days when injected into normal mice (Parsons, 1936, 1938, 1939). The active agent in these latter experiments must be of a microscopic nature and may possibly have the characteristics of a nucleoprotein—in short a virus. In these cases there is no appreciable latent period, for sarcomas can be observed on the 5th day after injection of the filtrate and are of a size comparable to a grafted sarcoma by the 15th day.

Further, if systemic changes consequent on the growth of a sarcoma in the body be due to an excess of nucleotide in the tumour

cells, the varying phases noted in the behaviour of sarcomas may depend on the amount of nucleotide liberated at different times. It has been observed (Parsons, 1938) that the lymph nodes of a sarcoma-bearing mouse have the faculty in a certain phase of the tumour of reproducing the sarcoma when grafted into a normal mouse. This faculty appears to be associated with marked malignancy of the growth, which in another phase is absent, when all attempts to reproduce the sarcoma from grafted glands fail. The grade of malignancy of a neoplasm may be proportional to the nucleotide changes in the tumour cells.

As the commercial mixture of pentnucleotides gave apparently contradictory results (see group 5 above), it was felt that the individual nucleotides should be examined. To this end experiments are in progress in collaboration with Professor J. M. Gulland and Dr G. R. Barker.

### SUMMARY

The action of a commercial mixture of the four pentose nucleotides of yeast nucleic acid on the tissues of experimental mice is described, with special reference to the similarity of the systemic changes induced to those accompanying the growth of sarcomas in these animals. These changes include amyloid infiltration of the liver and spleen.

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### Addendum

THE NATURE OF THE SUBSTANCE DEPOSITED IN THE SPLEEN AND LIVER  
OF THE MICE IN DR L. D. PARSONS' EXPERIMENTS

HUBERT M. TURNBULL

*From the Bernhard Baron Institute of the London Hospital*

In order to determine whether the hyaline substance deposited, after her experiments, in the spleen and less often the liver of mice was amyloid, Dr Parsons gave me several spleens and a few livers from CBA and stock mice with grafted tumours, and later two spleens from mice injected with a

mononucleotide isolated by Professor J M Gulland On the above material trials were made with different staining methods with the valuable collaboration of my head technician, Mr John King Dr Parsons' technician, Miss Downs had previously stained by the Congo red method of Letterer (1926) other spleens from mice injected with "pentnucleotide", and had shown all to Mr King and some to me The staining was definite but less deep and less red than in human material From its disposition and Congo red staining it was obvious that the deposit in the mice injected with "pentnucleotide" was the same as that in the material examined by us from the rest of the experiments

All the material had been fixed in 4 per cent saline formaldehyde and embedded in paraffin, and as a control we used a paraffin block of human liver similarly fixed from a case of "typical" lardaceous disease Stained sections were examined in daylight

The deposit in the spleen and less often in the liver of the mice has a disposition in both organs similar to that described by others in mouse amyloid It gave definitely the metachromatic reactions of human amyloid with methyl violet by Eden's method (Schmorl, 1928, p 211, Romeis, 1932, p 438) and with gentian violet by Schmorl's "undifferentiated" and "differentiated" methods (p 210) Compared with the control the staining of the deposit was less intense than in human amyloid, the stains faded much more rapidly in balsam, and in Schmorl's "differentiated" method the acetic acid differentiation usually had to be more rapid The older, inner part of the large ring like deposits round Malpighian bodies in the spleen withstood differentiation less well than the younger outer part of the rings and younger scattered deposits in the pulp The Congo red method of Freudenthal (1930 31, p 77) gave a stain usually slightly less red than in the human control, but in one instance as red Iodine reactions failed completely in both mouse and human tissue, all of which had been fixed in formaldehyde and embedded in paraffin This is not surprising, because all authors I have consulted place alcohol first in any list of fixatives for iodine reactions Schmorl says that it is best and Jaffé (1926) goes so far as to say that fixation in 83 per cent alcohol is necessary for all reactions in the mouse Further, Schmorl states in large print that paraffin sections "are not much good" for the iodine reactions Fortunately, Dr Parsons was able to provide us near the close of our investigation with half a small fresh spleen from a sarcomatous mouse This was fixed in absolute alcohol and cut upon the freezing microtome Only five sections were obtained and a small remnant retained Two sections were treated with dilute Lugol's solution and the deposit, in rings and semi-circles, was stained a typical very deep mahogany brown when examined with a hand lens and a paler brown when seen under the microscope The remaining three sections were treated with very dilute Lugol's solution for 30, 3 and 2 minutes respectively, and when mounted in distilled water sulphuric acid was drawn under the coverslip (iodine sulphuric reaction) The deposit gave respectively under the microscope a violet, a pink followed by violet and a grey colour Finally the small remnant was treated with Lugol's solution diluted until it was almost colourless The sulphuric acid changed the white Malpighian bodies to a pale blue tinged with green, but rings and horseshoes could not be detected Possibly in this thick piece of spleen the pale greenish blue of the deposit surrounding the Malpighian bodies was reflected through their centres No crystal like structures were seen in one spleen and two livers stained with Heidenhain's Azan (Romeis, 1932, p 411)

There can be no doubt, therefore, that the deposit in spleen and liver is mouse amyloid It has the characteristic distribution and gave methyl violet and gentian violet reactions and the Congo red reaction It also gave in the only suitably fixed and unimbedded piece of spleen a typical iodine reaction, but in the very few sections available for trial with the iodine sulphuric reaction gave colours which differed from Schmorl's pure blue

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PHAGOCYTOSIS OF VACCINIA VIRUS *IN VITRO*.\*

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(PLATES X AND XI)

IN 1906 Paschen described elementary bodies which he was able to stain in the fluid from vaccinia vesicles in great numbers before the liquid became purulent. In a letter to B Heymann in 1913 giving directions for staining, he mentioned that he had seen the vaccinia elementary bodies inside pus cells and concluded that phagocytosis of the virus had taken place.

While it is relatively easy to differentiate by staining virus bodies and inclusions in epithelial cells, it is more difficult to recognise them in leucocytes. The work of W Kolle (1899) showed that virus probably enters the blood stream and in 1909 von Prowazek and Yamamoto described phagocytosis in stained specimens of vaccinia. Von Waselewski (1905) had already indicated something of this sort, though his technique of using diluted acetic acid on unstained and unfixed specimens was open to criticism. In recent years vaccinia virus was found closely associated with leucocytes by Long and Olitsky (1929, 30, 1930), Smith (1929), Douglas and Smith (1930), Gildemeister and Hilgers (1930) and Sabin (1935), but, probably owing to the difficulty of differential staining, no descriptions of the virus within the leucocytes were given.

The method of prolonged observation which has been developed by the author and applied to viruses and bacteriophages gives a means of observing phagocytosis *in vitro*. The technique of prolonged observation has already been described, together with details of a number of special instruments necessary for continuous visual observation and photographic recording under the highest magnifications and making use of the highest possible resolution in dark ground illumination (Merling, 1935, 1938, 1940 *a* and *b*, 1941). For detailed description of methods and the use of the optical equipment reference must be made to these publications.

## TECHNIQUE

The technique employed in making the following observations was in the main identical with that described previously (Merling, 1940 *a* and *b*). The eyes of the rabbit were inoculated in the usual manner by scarification with the

\* Being part of a thesis approved by the University of London for the degree of Doctor of Philosophy.



same strain of vaccinia virus kindly made available by Professor McIntosh. After the vaccinal keratitis was well developed, which took about 3-6 days, thin tangential sections could easily be cut with a sharp knife, while of deeper layers scrapings could be detached with the blade of the knife. Control preparations from uninoculated corneae were of necessity of this type. The material obtained was placed in a drop of tyrode solution on a thin slide, covered with a coverslip and gentle pressure applied to expel excess of fluid. The coverslip was then sealed with paraffin wax in the usual way to prevent evaporation. Observations were carried out either at room temperature or on the warm stage of the microscope at 37° C. To obtain a clear view and avoid overcrowding of the field special care was taken to have only one or two small fragments of corneal tissue on any one slide. In this way up to twenty specimens could be prepared from one cornea. To prevent contamination during manipulation the best means, apart from the usual maintenance of sterile conditions, was found in speed of work.

Specimens were stained with Herzberg's Victoria 4 R blue and examined by means of a reflecting condenser for both transmitted light and dark ground illumination. The condenser provided easy verification and identification of the various cell constituents and inclusions. In this way the living cells were compared with dead cells dried in the air and embedded in oil of relatively low refractive index (oil of lavender,  $n_D = 1.462$ , and glycerin,  $n_D = 1.456$ ), with dead cells dried in the air and fixed and embedded in one or other of the media mentioned, and with stained specimens.

## RESULTS

As the keratitis develops, the number of leucocytes increases and on the sixth day the cornea is usually hazy. To gain an impression of the effect of the virus on leucocytes it was necessary to study the appearance and behaviour of leucocytes from a normal rabbit or one that showed slight conjunctivitis but had not been inoculated with vaccinia virus. Though it seemed at first very difficult to form a mental image of a "normal" leucocyte, this was later found to be easier by comparing it with the typical changes brought about by the virus. Therefore a detailed description of a normal leucocyte may be omitted, except to mention that all the usual constituents of leucocytes—nucleus in various lobes, granules of various sizes in movement, pseudopodia, filopodia, etc.—can easily be recognised with dark ground illumination. In the type of specimen described, "normal" leucocytes appear to be able to live up to five days, during which they exhibit various changes prior to death. Granules are normally in fast movement, which usually stops quite suddenly when the cell dies. Another indication of cell death is the droplet disintegration which appears to be a frequent but not invariable accompaniment.

Figs. 1 and 2 show the beginning and development of this process. After some experience had been gained of the appearance of "normal" leucocytes, it soon became obvious that in specimens from vaccinal keratitis there were great numbers of leucocytes showing intracellular virus. The question whether this appearance was due to phagocytosis of a passive virus or active invasion of the leucocytes by the virus



## PLATE X

FIGS. 1 and 2.—Two normal leucocytes showing different forms of droplet disintegration. Photographs taken within 10 minutes of removal from rabbit.  $\times 1000$ .

FIGS. 3-7.—Phagocytosis of vaccinia virus.  $\times 1000$ .

FIG. 3.—Centre, a leucocyte containing masses of intracellular virus: left upper quadrant, tissue cell with large empty extrusion; below centre, tissue cell with extrusion filled with moving virus (Brownian movement).

FIG. 4.—Four leucocytes: top right, normal, dead; second disintegrating; third normal, alive; fourth showing an extrusion filled with virus.

FIG. 5.—Leucocyte showing extrusion filled with virus.

FIG. 6.—Sheet of leucocytes, of which those to left of the ink line appear bright and contain intracellular virus; those to right of the line appear dull and contain no virus.

FIG. 7.—Five leucocytes. Note the difference in brightness between the central (virus-containing) cell and the surrounding cells which contain no virus. One cell is disintegrating.

PHAGOCYTOSIS OF VACCINIA VIRUS *IN VITRO*



FIG 1



FIG 2



FIG 3

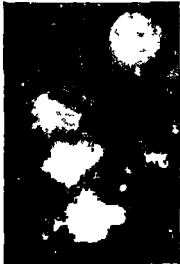


FIG 5



FIG 6



FIG 7



could fortunately be decided by observation of the actual process. There seems to be no doubt that phagocytosis of the virus took place in the majority of cells observed. But the virus did not become completely immobile when inside the leucocyte and obviously survived. Perhaps contrary to expectation, the virus formed colonies inside the leucocytes.

Figs. 3-7 show leucocytes with virus intracellular at the time the specimen was prepared. In fig. 3 there are shown several leucocytes and two tissue cells. One tissue cell has formed an empty extrusion, the other an extrusion filled with virus. Of the leucocytes pictured the one nearest the centre shows a white mass which represents rapidly moving virus. Fig. 4 shows four leucocytes, three of them uninfected. The one nearest the lower edge has formed a small lateral extrusion which is filled with rapidly moving virus. A similar picture is presented in fig. 15, which shows a leucocyte with a virus-filled extrusion at the top.

The differentiation of virus bodies from non-specific granules within the leucocytes is not very difficult. The virus bodies show the same characteristics within leucocytes as without, or in epithelial cell, or quite generally wherever they are found; sizes, shapes, brightness, colour, movement, etc., are constant and definite properties of the virus. Granules inside or outside leucocytes vary greatly in size, brightness, colour and movement. The immediately obvious difference between the two kinds of particles is in respect of brightness; living vaccinia virus bodies are about three times as bright as non-specific granules. Fig. 6 shows a sheet of leucocytes the greater number of which contain living and moving virus; a line drawn from near the upper right-hand corner to about the middle of the lower edge of the photograph has the infected (whiter) leucocytes to its left, the uninfected (greyer) ones to its right. Fig. 7 shows five leucocytes, of which the central cell contains virus, the left upper cell is disintegrating and the right upper cell shows an empty extrusion, while the two lower cells present no noteworthy details. The difference in brightness between the central cell and the others is apparent.

The number of virus bodies inside the leucocytes varied considerably. For the purpose of observing phagocytosis, leucocytes displaying movement of pseudopodia and filopodia were kept under prolonged observation. Phagocytosis was observed sufficiently often to make one satisfied as to its regular occurrence. A certain amount of chemotaxis was noticeable in the collection of active leucocytes around tissue cells which were discharging their virus content. Single elementary bodies were ingested in some instances within a few seconds. Small cysts containing virus were seen to be completely enveloped 2 or 3 minutes after having first been touched.

Having thus ascertained the existence of the process of phagocytosis of vaccinia virus the next question was the further fate of phagocytes

and intracellular virus. This question could only be answered by applying once more the principle of prolonged observation.

#### *Observations over a period of three days*

If a leucocyte containing virus persisted in its usual form and shape without breaking up, the virus in many cases formed colonies which were grouped around one of the nuclear lobes. The result was a striking resemblance to those intracellular colonies seen in epithelial and tissue cells—Guarnieri bodies—as they have been observed and recorded earlier (Merling, 1940a, figs. 11 and 12). The leucocyte shown in fig. 8 is apparently dead, three days after it had left the animal body, but it shows no signs of disintegration. The virus lies in the form of a crescent-shaped colony near the left edge of the cell. In fig. 9 a leucocyte is pictured which, after three days, has formed an extrusion filled with a small number of moving virus bodies. A few elementary bodies adherent to the membranous wall of the extrusion could be determined.

In fig. 10 a leucocyte (also three days old) is shown in process of bursting and releasing its content of virus. A similar picture is given in fig. 11 at a higher magnification. In all these leucocytes the intracellular colonies of virus show up well as bright areas. Particularly in fig. 11 the similarity to a Guarnieri body was very striking. Amongst the released virus particles all the stages of its life cycle can be found. Some leucocytes seemed to break up to a certain extent, discharging some of the virus. Parts of such cells remained unchanged while the virus colony grew very slowly amidst the debris. Fig. 12 shows three such partly broken up cells, with persisting virus colonies which had previously been intracellular. One of the cells has formed extrusions, to the surfaces of which some elementary bodies are adherent, resembling an appearance recorded in a previous paper (fig. 2, Merling, 1943).

#### *Observations over a period of three weeks*

After the specimens had been observed for three days it became clear that although the majority of the leucocytes were dead the development of the virus was not completed. Extracellular virus bodies remained mobile for several weeks, showing a slow increase in number. Intracellular virus bodies, if they were lying singly within the protoplasm of the cell, became motionless, but if they had formed colonies they continued to show the familiar fast movement of living inclusions. Fig. 13 is a photograph of the same three leucocytes as are shown in fig. 12 after an interval of three weeks. The position of the cells is slightly different owing to currents or possibly to amoeboid movement during the fourth and fifth days before the cells had died. In all three the virus colonies observed and recorded in fig. 12 had persisted and remained as mobile as on the first day.





## PLATE XI

FIGS. 8-12.—Photographs taken 72 hours after removal from rabbit.  $\times 1000$  (except fig. 11).

FIG. 8.—Leucocyte with intracellular virus. Movement has partly ceased.

FIG. 9.—Leucocyte showing extrusion filled with mobile virus (*cf.* fig. 2).

FIG. 10.—Leucocyte bursting and releasing virus.

FIG. 11.—Leucocyte disintegrating slowly and releasing virus.  $\times 1500$ .

FIG. 12.—Three disintegrating leucocytes containing intracellular virus, one showing an extrusion dotted with elementary bodies.

FIGS. 13-15.—Photographs taken 3 weeks after removal from rabbit.  $\times 1000$ .

FIG. 13.—Three leucocytes containing various amounts of moving virus.

FIG. 14.—Two leucocytes, of which the one on the right shows intracellular moving virus, that on the left is obscured by a droplet of fat.

FIG. 15.—Leucocyte which has formed one large droplet filled with moving and resting virus. Some cell constituents are faintly visible. *Cf.* fig. 1.

PHAGOCYTOSIS OF VACCINIA VIRUS *IN VITRO*



FIG. 8



FIG. 9



FIG. 10

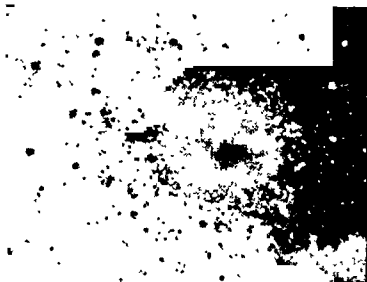


FIG. 11



FIG. 12



FIG. 14



FIG. 13

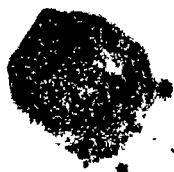


FIG. 15



The size of the colonies had increased and their outlines had become more distinct. It was not possible to have the three cells equally sharply focussed in fig. 13. Another cell with an intracellular colony three weeks old is shown in fig. 14. By the side of the cell are lying some droplets, one of which is so bright that its outline could not be recorded at an exposure time suited to the cell and colony. This very bright droplet of fat shows only as a diffraction image. The faultlessness of the diffraction rings incidentally furnishes a severe test of the quality of the optical image.

Another instance of colony formation in a leucocyte following phagocytosis is given in fig. 15. Here the leucocyte can no longer be recognised as such, though some cell constituents are still faintly visible. The whole cell seems to be blown up like a balloon and it is filled with fast-moving virus; resting virus is adherent to the membrane. In visual observation it would be very difficult to distinguish between a cyst and an image like that shown in fig. 15. Only prolonged observation and the following and recording of developments can supply all the data necessary for differentiation of this kind.

The ultimate end of these colonies within dead leucocytes was similar to that previously described for colonies formed under different circumstances (Merling, 1940*b*, 1943). With the break up of the cell or its debris or the bursting of an extrusion the greater number of virus bodies were released. Gradually and slowly all movements came to a halt and no further changes occurred. Virus bodies adherent to a glass surface steadily lost brightness but never disappeared completely. Such specimens were frequently opened and stained. They then furnished further confirmation of the appearances described, though within such limitations of staining methods as inferior resolution, fixation, artefacts, etc. Delicate structures like the one shown in fig. 15 usually did not stand drying, even when this was carried out in the most careful way in a moist chamber. In most cases they shrivelled up into a mass that showed no resemblance to their former appearance, as was easily observed with dry objectives (8 mm. and 4.2 mm.) after removal of the coverslip. Further manipulation like fixing and staining appeared to produce less change.

#### DISCUSSION

Under the conditions of the experiments vaccinia virus can be recognised in the living leucocyte as soon as some experience has been gained in differentiating virus from normal cell constituents. Mitochondria caused some difficulty at first, but later, when attention was constantly focussed on all their characteristics and those of the virus, no confusion arose. The question of invasion of the leucocytes by the virus or its phagocytosis (active ingestion) was decided by direct observation. The most remarkable fact which came to light

in these observations was the likeness of the colonies derived from leucocytes to those originating in epithelial cells. No essential difference has ever been recorded. If there had been any need for further confirmation that it was the virus which produced all the appearances here described, it is clearly provided by the fact that two phylo- and ontogenetically different types of cells—epithelial cells and leucocytes—formed the matrix for a third unknown, supposed to be a colony of a micro-organism. Both types of cells showed the same changes and appearances following the intracellular presence of the virus.

The importance of the discovery of viral phagocytosis need not be emphasised here. It need only be mentioned as an immunological phenomenon affording one more fact connecting viruses with larger micro-organisms. Phagocytosis of bacteria has formed a valuable tool in bacteriological technique almost since the days of its discovery by Metchnikoff, and the knowledge that the same process takes place with viruses may also prove to be of value.

#### SUMMARY

The method of prolonged observation has been applied to the study of the relationship of leucocytes and vaccinia virus. It was possible to take photographs which show the virus living intracellularly. Intraleucocytic virus did not die but formed colonies which survived the death of the leucocyte and continued to grow. These colonies appeared in no way different from those originating from epithelial cells or from a lawn of virus bodies as described in previous publications.

I am indebted to Professor James McIntosh for his valuable advice and criticism and to the Middlesex Hospital Medical School for facilities in carrying out this work.

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## THE BACTERIAL FLORA OF WOUNDS AND SEPTIC LESIONS OF THE HAND

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THIS communication reports the data (to which our colleagues, Miss B Clayton-Cooper and Mr G J Harper, have contributed) from a bacteriological survey of some 1200 clean and septic wounds and other septic lesions, made to determine the relative importance of the various bacterial species as causes of sepsis in the very common small wounds of the hand resulting from industrial and domestic accidents

### BACTERIOLOGICAL TECHNIQUE

The frequency with which various organisms are found in a given site depends in part on the bacteriological technique employed and for this reason we have set out and analysed our technique in some detail, it was adapted from that recommended in the Medical Research Council War Memorandum no 2 (1943)

*Sampling methods* The efficiency of any sampling method is measurable only in terms of the optimum cultural technique for each bacterial species present and for each variation in the material under test. There is no simple method that is optimal for all circumstances and species and some degree of compromise is necessary. The methods available for sampling wounds include (1) the swab, (2) the excision of fragments of wound tissue, advocated by American workers, *e.g.* Pulaski *et al* (1941), (3) the collection of wound exudate, and (4) rinsing the wound with a small quantity of nutrient broth. The wounds studied by us seldom had any exudate and were too small for rinsing. As a routine we used a swab, moistened if necessary with peptone water, and whenever possible cultivated excised tissue fragments in addition. A comparison of the frequency of obtaining *Staph aureus* by these two methods in 228 small wounds showed that each alone yielded about the same proportion of positives—51/228 and 60/228 with a mean of 54.5/228—that is, there is little to choose between them. The proportion of positives by the combined methods was 95/228, about double the mean value for a single sample. These results are most simply explained by assuming that the niduses of staphylococci in the samples are exceedingly scanty, being present on the average once in two or three samples, whether swabs or pieces of tissue. Excised tissue was available, however, only from fresh wounds, and for healing wounds we relied on the swab, though some infections will remain unrecognised by this method. Nevertheless the error from this source may be assumed constant and comparisons between different groups of wounds sampled by the same technique will be valid. Excised tissue was collected in about 1 c.c. of 0.05 per cent liquid broth, to neutralise as far as possible the antibacterial substances present in the tissue fluids (on Haebler and Miles, 1938).

In the early stages of the investigation, swabs from 27 fresh wounds and 87 septic wounds were examined by an extended technique in which a more thorough study was made of the anaerobic plates, and the cooked meat cultures were subcultivated a second time after 7 days' incubation. The frequency of the bacterial species isolated did not differ significantly from those recorded in table III, except that a slightly larger proportion of *Cl. welchii* and aerobic spore-bearing bacilli was obtained.

*Neutralisation of bacteriostatic agents present in samples.* For samples from wounds undergoing sulphonamide therapy, *p*-amino benzoic acid (0.05 mg. per 100 c.c.) was incorporated in plates (M.R.C. War Memo. no. 2). The appropriate amount of penicillinase-containing paracolon powder (Harper, 1943) was added when samples were expected to contain penicillin.

The dilution of the swab in the 10 c.c. of the cooked meat medium is sufficient to overcome the inhibition due to small quantities of drugs. Moreover, the broth itself contains some substance, as yet not clearly identified, which antagonises the action of the sulphonamides (Macleod, 1940; Harper and Cawston, 1945), so that staphylococci will grow in it in the presence of 50 mg. of sulphathiazole per 100 c.c. Of the common wound antiseptics, gentian violet and flavine, in amounts greater than are likely to be present on a dye-laden swab from a wound, are also neutralised in the meat medium, probably by absorption, since the supernatant broth becomes decolourised.

*Methods of identification of various organisms.* We have reserved the term *Staphylococcus aureus* for coagulase-positive staphylococci and have designated all staphylococci and miscellaneous gram-positive cocci which fail to show coagulase activity as "micrococci". Coagulase tests were made by the methods of Cadness-Graves *et al.* (1943). The term *Streptococcus pyogenes* has been reserved for group A hæmolytic streptococci, grouped by a modification of Lancefield's method (Williams and Harper, 1944).

Streptococci of *viridans* type were distinguished by morphology and by hæmolysis on the blood agar plate. All aerobic gram-negative bacilli of the coliform type were classified by fermentation and other biochemical reactions into species of the genera *Bacterium*, *Proteus*, *Pseudomonas*, *Chromobacter*, *Achromobacter*, etc. *Cl. welchii* was identified by the Nagler plate reaction (Hayward, 1943) and by its production of a stormy clot in iron-milk (M.R.C. War Memo. no. 2). Other anaerobic organisms were identified by Miss N. J. Hayward, University College Hospital Medical School, London.

## BACTERIAL FLORA OF WOUNDS

It is convenient to distinguish "*sepsis*", as a clinical diagnosis based on the signs of inflammation in the wound, from "*infection*", a bacteriological diagnosis based on the results of culture. All but some 8 per cent. of septic wounds are demonstrably infected with pyogenic organisms (see below), but up to 40 per cent. of clinically healthy wounds are also found to be infected. The latter condition we term "*silent infection*". We have used the term "*contamination*" to refer to the presence of bacteria in wounds less than 6 hours old, for within this period neither the bacterial invasion nor the stimulation of tissue responses, which together constitute "*infection*", can, in the absence of clinical signs, be presumed to have taken place.

Table III shows the bacteriological findings in various categories of wounds. In column 1 are fresh small cuts of the hand and forearm, sampled, and treated by trimming and suture, within 6 hours of the

injury. A wound swab and the excised tissue were cultured in these cases. A number of the wounds were observed until healed; column 2

TABLE III

*The bacterial flora of wounds and septic lesions*

Organism	Percentage frequency of isolation in			
	432 fresh lacerations of hands (1)	278 healing lacerations, never septic* (2)	142 septic lacerations (3)	345 other septic lesions (4)
<i>Staph. aureus</i> . . .	21.1	37.0	82.3 (90.1)†	88.2
<i>Strep. pyogenes</i> . . .	1.2	3.2	17.6	26.4
Coliform organisms . . .	3.7	7.9	4.0	2.3
<i>Cl. welchii</i> . . .	3.2	10.4	4.2	0.6
Micrococci . . .	85.4	89.6	25.4	11.0
Aerobic spore-bearing organisms	9.0	19.1	6.3	1.7
Diphtheroid organisms	4.9	6.8	1.4	2.0
<i>Strep. viridans</i> type organisms	6.3	2.9	1.4	0.3
Hæmolytic streptococci, not group A	0.7	0	0.7	1.2
Miscellaneous Clostridia . . .	0	0.4	0	0.3
<i>H. influenzae</i> . . .	0	0	0	0.3
Sterile . . . . .	6.7	2.5	1.4	2.0‡

\* Figures derived from consideration of all follow-up swabs received from any one patient, ranging up to 7 (mean, 2.18)

† Including data from subsequent swabs of wounds showing no pathogens in the first swab

‡ Three of the 7 wounds later showed *Staph. aureus*, 2 showed micrococci and 1 an aerobic spore-bearing organism

shows the bacterial flora isolated from those that never showed sepsis, and column 3 similar data for the septic wounds of the same series and from other larger septic wounds also initially treated by trimming and suture. Column 4 shows the bacteriological findings in a group of wounds with established sepsis when they were first presented at the hospital. The majority of these wounds were sampled at the time of operation.

In table IV the same wounds are analysed in greater detail, taking account only of the presence or absence of *Staph. aureus* and *Strep. pyogenes*.

The organisms most frequently isolated from fresh wounds were micrococci, but 21 per cent. yielded *Staph. aureus* in small numbers. The carrier rate for *Staph. aureus* on the normal skin of the wrist is about 10-20 per cent. (Miles *et al.*, 1944) and data from 320 patients with fresh small cuts of the hand show that 31 per cent. of the 30 who carried *Staph. aureus* on the back of the wrist of the uninjured hand also had contaminated wounds, compared with 17 per cent. of the 281 non-carriers. This is an association that might have arisen by chance in about 5 per cent. of trials. The association is much



closer when the skin near the wound is sampled. Table V shows that our results for the proportion of wounds contaminated with *Staph. aureus* and *Strep. pyogenes* are in accord with those of other workers.

TABLE IV

*The frequency of Staph. aureus and Strep. pyogenes in wounds and septic lesions*

Type of wound	No. of wounds	Percentage yielding			
		<i>Staph. aureus</i> only	<i>Strep. pyogenes</i> only	<i>Staph. aureus</i> and <i>Strep. pyogenes</i>	Neither <i>Staph. aureus</i> nor <i>Strep. pyogenes</i>
Minor lacerations, less than 6 hours old	432	20.6	0.7	0.5	78.2
Healing minor lacerations, never septic *	278	35.6	1.8	1.4	61.2
Minor lacerations, septic less than 6 days †	55	67.3	0	1.8	30.9 ‡
Minor lacerations, septic more than 5 days †	38	89.4	0	5.3	5.3 §
Septic lacerations, major	49	51.0	6.1	38.8	4.1
Suppurative tenosynovitis	37	58.8	18.9	8.1	16.2 ¶
Pulp space abscesses	111	92.0	2.6	1.8	3.6
Paronychia	67	29.8	13.4	52.2	4.6
Web space abscesses	22	86.4	0	13.6	0
Miscellaneous septic lesions of hands	108	68.5	3.7	23.2	4.6
Total septic lesions and septic lacerations	487	68.2	5.4	18.4	8.0 **

\* Figures derived from consideration of all swabs from any one wound.

† Sampled within 2 days of the discovery of sepsis.

‡ 8 of the 17 wounds showed *Staph. aureus* in later swabs.

§ 1 " " 2 " " " " " "

|| All sampled at the time of first surgical intervention.

¶ 3 of the 6 wounds showed *Staph. aureus* in later swabs.

\*\* 12 " " 39 " " " " " " "

The infection rate among the clinically healthy healing wounds which never showed clinical sepsis was nearly double the *Staph. aureus* contamination rate in fresh wounds, and the degree of infection, judged by the growth on direct plates from the swab, was often as great as that from frankly septic lesions.

The great majority of septic lacerations and other septic lesions yielded *Staph. aureus* or *Strep. pyogenes*, and in general it was only those wounds that were septic for a short time which failed to yield pyogenic cocci. Another notable fact is the high prevalence of staphylococcal as compared with streptococcal infections. In only three types of wound are streptococcal infections at all common: suppurative tenosynovitis (27 per cent.), paronychia (65 per cent.) and septic major lacerations (45 per cent.). It is possible that the high prevalence of streptococcal infection in the two latter is the

result of added infection, for the initial infection of small wounds of the hand is usually staphylococcal (unpublished studies) and both paronychia and major lacerations are peculiarly liable to added infection. The septic major lacerations were moderately large wounds which had demanded surgical repair at the time of injury.

TABLE V

*The bacterial flora of fresh and "old" wounds*

Reference	Sampling method: S = swab T = tissue	Age of wounds when sampled	No. of cases	Percentage of wounds yielding			
				<i>Staph. aureus</i>	<i>Strep. pyogenes</i>	<i>Bact. coli</i> type	<i>Ps. pyocyanea</i>
Pulaski, Meleney and Spaeth (1941)	T	24 hrs.	200	18.8 *	17.0 †	23 ‡	
Spooner (1941b)	S	48 "	31	6.5	6.5	19.0	0
Hare and Willits (1942)	S	2 "	187	18.0	...	...	...
De Waal (1943) §	S	10 "	342	16.9	(16.7) †	3.0	0.3
Altermeier and Gibbs (1944)	T	3 "	99	*7.0	(13.0) †	20 ‡	
Miles <i>et al.</i> (1940)	S	1-40 days	105	54.3	31.4	24.7	7.6
Spooner (1941b)	S	2-21 "	13	69.2	38.4	30.8	7.7
"	S	>35 "	29	65.6	69.0	51.7	27.6
Pulvertaft (1943)	S	...	52	54.4	36.6	19.2	11.5
De Waal (1943)	S	...	708	32.8	20.6 †	23.6	12.4
Grinnell (1937) ¶	...	...	112	46.4	51.8 †	0	0

\* Estimated from the proportion of coagulase-positive staphylococci found by the authors in the minority of their wound staphylococci tested for the enzyme.

† "Hemolytic streptococci", not definitely *Strep. pyogenes*.

‡ "Enterobacteria".

§ Wounds of hand and forearm only.

|| "Hemolytic and non-hemolytic *Staph. aureus*", coagulase activity not recorded.

¶ All cases of suppurative tenosynovitis, i.e. definitely septic.

Many had been open discharging wounds for some time before swabbing. Paronychia is frequently a chronic infection which may remain open and untreated for some time. On the other hand, the wounds showing a very high prevalence of pure *Staph. aureus* infection—the pulp space abscesses—are those in which the lesion often arises from a puncture wound, which, very soon after the injury, is effectively sealed off from the exterior and from the possibility of secondary infection.

The association of streptococci with open wounds is also demonstrable in the "miscellaneous" group. Eighty-eight of these wounds for which adequate records are available can be divided into two groups: 54 wounds which were noted as "open and discharging" at the time of sampling, and 34 which were made by operations on closed abscesses and were sampled at operation.

Streptococci were present in 44.4 per cent. of the "open" wounds, but in only 8.8 per cent. of the "closed". This is a highly significant difference, being one that might be expected to occur by chance less than once in 10,000 trials ( $\chi^2 = 17.16$ ).

We have attempted to classify 25 of the 36 strains of coliform organisms isolated from these wounds. The frequency of isolation of the different species was as follows:—*Bact. coli*, 7; *Bact. aerogenes*, 3; *Achromobacter* 4; *Ps. pyocyanea*, 4; *Proteus morgani*, 3; and paracolon bacillus, 1. Four strains could not be classified. None of the wounds yielded *Proteus vulgaris*.

### DISCUSSION

A number of reports are available dealing with the bacteriology of major war and civilian wounds at varying intervals after infliction. Some of these are compared in table V, though it must be emphasised that the species frequencies are reflections of technique and are not strictly comparable. The table shows a much greater proportion of coliform infections than we have observed. Our wounds, however, were exclusively industrial and domestic wounds of the hand. Those reported by other workers included wounds of all parts of the body, and De Waal (1943) has shown that wounds of the thigh and abdomen show a much higher incidence of coliform contamination than do those of the forearm and hand. Our technique was inadequate for the isolation of slowly growing or fastidious organisms such as many of the Clostridia, *Haemophilus*, etc., but it is almost certainly optimal for the isolation of coliform organisms, whose absence must therefore be attributed to the difference in the population of the wounds studied. This conclusion is supported by the results of applying the technique to war wounds of the lower limb: fifty-six such wounds 3-8 days old were recently investigated and 27 (48 per cent.) yielded coliform organisms.

Various explanations may be offered for the finding that a small proportion of septic wounds fail to yield pyogenic cocci.

1. The changes noted in the wound and interpreted as sepsis may not in fact have been the result of bacterial invasion.

2. The sampling may not have been adequate, or viable bacteria may have been present only in the deeper layers of the wound. In some wounds, however, mild sepsis was noted for a day or more before cultures yielded *Staph. aureus* (see footnotes to tables III and IV). It may be that the micrococci isolated were responsible for the initial changes and that the staphylococci were added from without to a wound opened up by micrococcal infection. Or the sepsis may have been due all the while to *Staph. aureus*, which we failed to isolate until a late stage. In this connection it is of interest that cultures taken at the time of first incision from a few abscesses and septic tendon sheaths have proved sterile, though they yielded a profuse growth of *Staph. aureus* on the following day. It is possible

that while viable organisms are still present in the wall, the pus in a mature abscess can in fact be sterile, perhaps as a result of the activity of the leucocytes (Fleming, 1919-20), and perhaps because the organisms do not easily flow into the pus against the hydrostatic pressure in the abscess cavity.

3. The sepsis may have been caused by infection with microbes not easily identified by the cultural method used, *e.g.* certain *Clostridia*, members of the *Bacteroides* and *fusiformis* groups, anaerobic streptococci, non-haemolytic group A streptococci, *Hæmophilus*, etc.

4. The organism isolated, though usually regarded as non-pathogenic (*e.g.* coagulase-negative staphylococci, etc.), may have been responsible for the disease process in these wounds. We have observed wounds which were without doubt septic, though careful and repeated cultivation on various media yielded a pure growth of micrococci only. Unfortunately we have not been able to test the virulence of these organisms in animals.

It is probable that all these explanations are relevant to some cases of sepsis, but, as table III shows, the problem is not numerically important and sepsis in wounds of the hand can be accounted for in the large majority of cases by infection with *Staph. aureus* or *Strep. pyogenes*.

#### SUMMARY

A bacteriological survey (mainly in terms of aerobic bacteria) has been made of 1197 wounds and septic lesions of the hand and the results classified according to the nature of the lesion. *Staph. aureus* and *Strep. pyogenes* predominated as the chief causes of infection.

*Staph. aureus* was isolated from 21 per cent. of 432 fresh wounds, from 37 per cent. of 278 apparently healthy healing cuts, from 82 per cent. of 142 clinically septic cuts and from 88 per cent. of 345 other septic lesions. The corresponding percentages of wounds yielding *Strep. pyogenes* were 1, 3, 18 and 26. Only 39 (8 per cent.) of 487 septic lacerations and other septic lesions failed to yield *Staph. aureus* or *Strep. pyogenes*, though 12 of these yielded *Staph. aureus* in later samples.

Bacteria of presumed faecal origin were rarely found in these wounds of the hand.

The majority of infections of the hand that develop as closed lesions, *e.g.* pulp space abscesses, are due to *Staph. aureus*; those that develop as open lesions, *e.g.* paronychia and large septic lacerations, commonly yield *Strep. pyogenes* as well. This difference suggests that *Strep. pyogenes* wound infections result from long exposure to a relatively low risk, and that many of them are added after the infliction of the wound.

We are indebted to Mr William Gissano, clinical director of the Birmingham Accident Hospital, and to the staff for their co-operation in this survey.

Part of this work is incorporated in a thesis submitted by R. W. and approved by the University of London for the degree of M.D.

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# A STUDY OF THE EFFECTS OF APPLYING PRESSURE TO EXPERIMENTAL THERMAL BURNS \*

G. R. CAMERON, J. W. ALLEN, R. F. G. COLES and J. P. RUTLAND

THE striking results obtained by Cope and Rhinelander (1943) in treating burns by the application of pressure has led to much interest in this therapeutic measure. We record experiments designed (1) to assess the value during the first 24 hours of the prompt application of pressure to extensively burnt limbs and (2) to clarify our knowledge of the physiological principles involved in such treatment.

## METHODS

Male and female goats, 15-43 kg. body weight, were employed. Females were preferred because of the awkward arrangement of the external genital organs and the unpleasant habits of the bully. Pregnant females were avoided whenever possible and placid, well nourished animals, free from anaemia or obvious lung disease were chosen. Twenty eight goats in all were used, 14 of which were treated with pressure, 14 being burnt but not treated. To economise space we give details only of the 12 experiments with severe burning. The full charts and tables have been deposited with the Librarian, General Library, British Museum (Natural History), London, S W. 7.

*Blood examination.* Since we are not aware of any thorough investigation of the normal goat's blood picture (Barcroft *et al*, 1919-20, give figures for

TABLE I

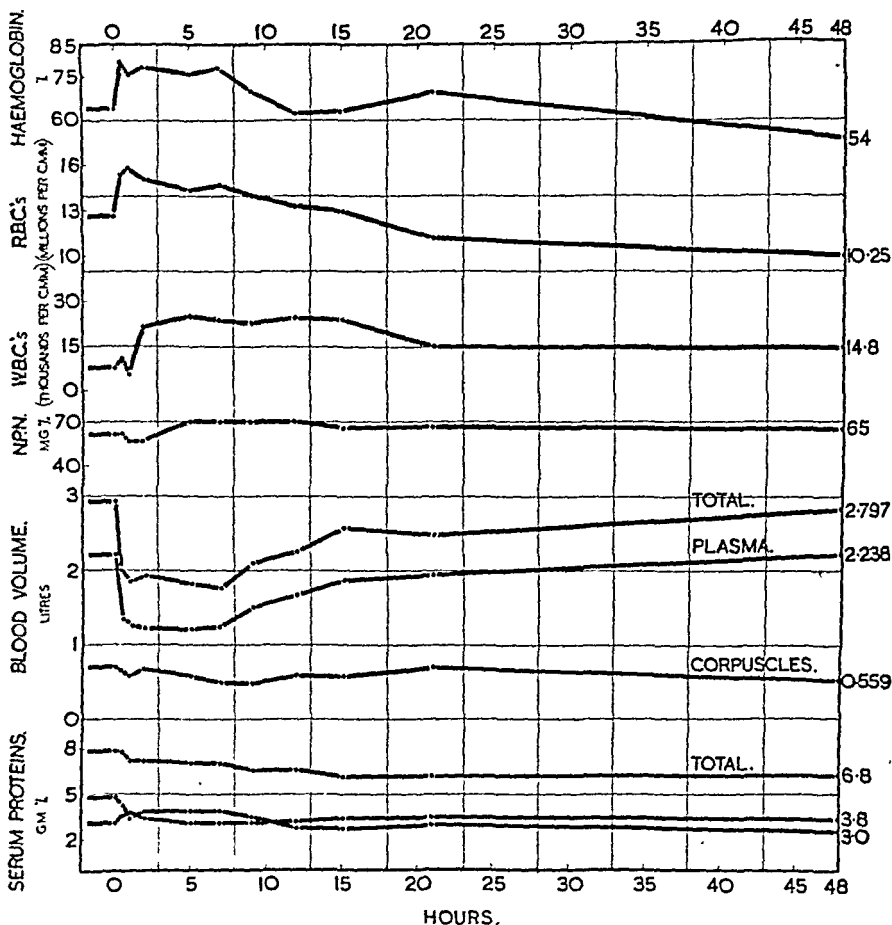
*Normal goats. Summary of blood component means with statistical constants*

Component	Mean	Standard deviation ( $\sigma$ )	Standard error (S.E.)	No of goats examined
Hæmoglobin (percentage)	68.4	12.2	1.4	83
Red corpuscles (millions per c mm)	12.49	2.18	0.26	70
White corpuscles (thousands per c mm)	10.28	3.12	0.28	78
Hæmatocrit (percentage)	23.75	5.96	0.81	54
Total blood volume (c.c. per kg body weight)	65.34	10.08	1.31	59
Plasma volume (c.c. per kg body weight)	49.41	9.26	1.21	59
Cell volume (c.c. per kg body weight)	15.93	3.02	0.51	59
Total serum proteins (g. per 100 c.c.)	8.55	1.32	0.17	57
Serum albumin (g. per 100 c.c.)	3.54	0.72	0.10	57
Serum globulin (g. per 100 c.c.)	5.18	1.25	0.17	57
Non protein nitrogen (mg. per 100 c.c.)	47.46	11.74	1.56	57

hæmoglobin) and blood volume we include a summary of our findings in such a series (table I). A curious feature is the high globulin content of the serum

\* Report to the Burns Sub Committee of the Medical Research Council's War Wounds Committee

as determined by salting-out with 22.5 per cent.  $\text{Na}_2\text{SO}_4$  at  $38^\circ\text{C}$ . Haemoglobin was estimated by the Haldane CO method, red and white cells were counted in a Thoma chamber after dilution with the usual diluting fluids (Hayem's fluid often gives agglutination of goat's red corpuscles but the saline-citrate-formalin mixture described by Whitby and Britton (1939) is free from this fault) and for haematocrit determinations 10 capillary tubes were used, with centrifugation at 3000 *r.p.m.* for 45 minutes. Serum proteins and non-protein nitrogen



Thermal burn. Blood changes in goat: 15.6 per cent. of body surface scalded at  $86^\circ\text{C}$ . for  $2\frac{1}{2}$  minutes.

were estimated in oxalated blood by micro-Kjeldahl digestion and direct nesslerisation, albumin and globulin being separated by the precipitation of globulin with 22.5 per cent. of  $\text{Na}_2\text{SO}_4$  kept at  $38^\circ\text{C}$ . Non-protein nitrogen was obtained after precipitating the proteins with trichloroacetic acid. Plasma volume was determined by the method of Kennedy and Millikan (1938) and Courtice (1943-44), employing the blue dye T. 1824, which can be estimated with great accuracy by the aid of a photo-electric cell. The device introduced by Harington, Pochin and Squire (1939-42) for overcoming opalescence of plasma proved valuable. For repeated estimations in the same animal a sample of plasma was withdrawn before each new injection of dye to serve as a control. Cell volume was calculated from the haematocrit and plasma volume

and the total blood volume obtained by addition. This procedure of course assumes that values from venous (jugular) blood are fair indications of the total blood distribution, an assumption not necessarily correct when there is serious circulatory disturbance. For that reason we place no emphasis on total blood and cell volumes but merely record them in the protocols, the plasma volume is the useful observation. Other sources of error in the dye method and its applicability to conditions of abnormal capillary permeability are discussed in the thoughtful paper of Courtice and the publications of Gibson and Evans (1937), Gibson and Evelyn (1938), Price and Longmire (1942), Gregerson and Rawson (1942-43) and King *et al* (1942-43).

**Burning** For burning we placed goats in oil drums containing water at 86° C., so that the fore and hind limbs rested in the water to a known level, for 2½ minutes. Nembutal, 15 mg per kg, was administered intravenously to eliminate pain and struggling. Such a technique gave remarkably constant burns involving about 20 per cent of the total body surface and extending about midway through the dermis. The burnt areas were measured by applying flexible paper strips and the percentage of total body surface calculated from the formula  $S = 0.115 W^2$ , where  $S$  = body surface in square metres,  $W$  = body weight in kg. We obtained this formula by direct measurement of a few goats, it is very like the formula given by Meeh (1879) for dogs and man.

**Pressure application** Quick setting "Cellona" plaster bandages were applied to the burnt areas at once and in a few experiments 4 and 6 hours after burning. In most cases the bandages were applied fairly tightly so as to raise the subcutaneous tissue pressure by 5-7 mm Hg as estimated by the method of Burch and Sodeman (1937). We carried out a number of estimations of subcutaneous pressure in normal human subjects, obtaining results similar to those of the American workers, and then extended the method to tissues enclosed in plaster bandage. Reproducible results were obtained in the same subject. Goat figures were also fairly constant. In other experiments skin tight plaster was applied, much less pressure being exerted on the underlying tissues. It was essential to apply the bandages with considerable overlap beyond the burn margins since oedematous fluids tend to be squeezed some distance from the burn by the bandages.

**After care** This is important if serious shock is to be prevented. The goats were kept warm by means of blankets and hot water bottles in straw lined pens, receiving food and water if they wished it. Bleeding was carried out expeditiously and so as to remove minimal amounts of blood consistent with the investigation. Keeping the animals quiet and free from fright was essential, we came to dread the bleating restless goat as a likely candidate for shock and death.

**Other observations** Throughout the experimental period repeated observations were made on clinical behaviour, respiratory rate, rectal temperature, passage of urine and the condition of the neck veins, blood pressure estimations could not be included in our scheme. Twenty four hours after burning the animals were killed with Nembutal and autopsy was at once conducted. The skin and subcutaneous tissues down to the underlying skeletal muscles were dissected out and weighed, the limbs severed at the burn margins and weighed and a careful search made for fluid loss into serous cavities and lungs. The lung:heart ratio, a useful index of pulmonary oedema, was calculated. Material from main organs except the brain was fixed in 10 per cent formol saline and in Susa for burnt skin, paraffin and frozen sections being stained with Ehrlich's acid haematoxylin and eosin, Weigert's iron haematoxylin and van Gieson and Scharlach R for fats. The prussian blue method was used for the spleen and Heidenhain's azan method for kidneys and burns. The bladder urine was examined for blood cells and pigment, casts, albumin and sugar.



## RESULTS

*The course of an extensive untreated burn*

An uncomplicated thermal burn runs a constant course in the goat and shows close similarity to the course in man. Within half-an-hour of burning about 20 per cent. of the total body surface much plasma leaves the vessels of the affected region. This results in a rapidly increasing hæmoconcentration, a speedy fall in plasma and total blood volume with an unchanged, sometimes increased, cell volume and a steady decline in the serum protein concentration (fig.). The globulin is often affected more than the albumin, a feature we have noticed in the goat in other conditions with increased capillary permeability. For the first 4 hours these changes are pronounced, next comes a period of about 4 hours in which little change occurs and then at the 8th or 9th hour recovery sets in. At 24 hours hæmoglobin, hæmatocrit and red cell counts are normal or subnormal, blood and plasma volumes remaining depressed. Serum proteins have seldom reached the initial values by this time. Local cedema at the burn site increases during the first 24 hours or longer but begins to subside shortly afterwards. Anæmia, usually mild, may then develop; it does not seem to be due to blood dilution. Serum proteins recover in about 3 days. Leucocytosis appears within a few hours of burning and often persists for many days. Clinical features are not pronounced during the first day; the rectal temperature may show a slight rise and the respiratory rate may increase for a few hours at a time when circulatory disturbance can be detected. If the animal is restless or in a state of advanced pregnancy or anæmic these features may be pronounced and death sometimes occurs at 4-6 hours. Fall in rectal temperature has not been met with even though some animals were very shocked.

Pathological changes are slight. Animals killed 24 hours after extensive burning show lung hæmorrhages, especially along the postero-medial margins, and sometimes recent endocardial and pericardial hæmorrhage, congestion of the lungs, liver, rarely spleen and duodenal mucosa and occasionally severe cedema of the bone marrow. Renal changes are slight and may be associated with leakage of small amounts of altered blood through the glomeruli. Pigment casts may be forming at this stage. Erythrophagocytosis is striking in the spleen. Hæmolysis and sometimes hæmoglobinuria are found during the first 12 hours but are slight and have not led to jaundice in our animals. Spherocytosis and "budding" of red corpuscles may be seen shortly after burning. The adrenals and other ductless glands present no abnormalities. Slight liver damage occurred in one goat. Cerebral cedema was not obvious in 3 animals whose central nervous system was carefully examined. None of these pathological findings can be viewed as alarming and we doubt whether they are associated with serious disturbance of function. We have

formed the opinion that there are no changes in the organs after extensive burning of the extremities other than can be accounted for by the plasma loss into the burnt area.

*The effects of applying pressure to burns*

The results of our experiments are in part set out in tables II and III and summarised in table IV. It seems that prompt application of pressure reduces maximal percentage hæmoglobin rise (mean of  $11.18 \pm 1.87$  in pressure-treated goats against  $33.17 \pm 3.71$  in untreated) and percentage plasma volume fall (mean of  $10.83 \pm 2.09$  against  $31.33 \pm 3.03$ ), whilst it encourages the return to normal of the plasma volume. It will be noted that from time to time cell volume has increased, especially in the early hours after burning, both in treated and control goats. We attribute this to splenic contraction, the spleen being a rather large organ in the goat. Serum protein loss from the blood is also decreased by pressure treatment. The mean maximal fall of serum protein in treated goats was  $10.83 \pm 1.39$  per cent., for untreated animals  $20.0 \pm 1.71$  per cent. All these differences are statistically significant.

In table III we have collected data about the weights of the total burnt limbs and skin in both series of animals. There are certain obvious criticisms of the method. Pressure treatment leads to extension of œdema beyond the burn margins, sometimes for a distance, hence comparisons of total burnt limbs, *i.e.* limbs cut off at the burnt margins, may be of restricted value. Comparison also requires (1) similar areas being burned, (2) no gross differences in weight of limbs compared. It is not easy to adjust these difficulties satisfactorily, for rarely can animals be selected of exactly the same body weight and build. We accordingly present our results along with notes which indicate whether a fair comparison may be made, but we make no attempt to draw any quantitative conclusions. We believe this evidence shows that pressure has reduced the œdema in the burnt area and it is our impression that reduction is not inconsiderable. The local œdema figures are the more impressive because there was no fluid loss from the burns by "weeping" or blistering, phenomena not encountered in the burnt goat. Nor did there appear to be loss in other ways, for serous effusions were not seen, pulmonary œdema was not discovered and none of the animals developed diarrhœa. We doubt whether the inconstant and usually transient quickening in respiratory rate could have influenced fluid exchange very much.

We also gained the impression that on the whole treated animals showed less severe clinical features than untreated, although temperature and respiration records did not suggest any notable difference. Pathological examination, too, was of little assistance in assessing the value of treatment, since both groups of animals showed little systemic injury.

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TABLE II

*Blood picture, blood volume, serum proteins, non-protein nitrogen, respiratory rate and rectal temperature in representative pressure-treated and untreated goats*

Time interval	Hb. (per cent.)	R.B.C. (millions per c.mm.)	Hematocrit (per cent.)	Blood volume (c.c.)			Serum proteins (g. per 100 c.c.)			N.P.N. (mg. per 100 c.c.)	Resps. per min.	Rectal temp. (°F.)
				Total	Plasma	Cell	Total	Albumin	Globulin			
Goat 65. Male. Body weight 28 kg. Body surface 10,004 sq. cm. Percentage surface burnt 21.3. Pressure for 24 hours.												
Before burning	72	10.05	23.3	2207	1762	535	9.87	3.75	6.12	36	19	103.0
One hour after burning	74	10.44	24.3	2298	1740	558	9.78	3.46	6.32	35	21	103.9
2 hours "	74	10.76	24.2	2295	1740	555	9.50	3.49	6.01	36	21	103.7
4 "	78	11.99	24.6	2225	1678	547	8.81	3.49	5.32	36	20	103.5
6 "	77	11.73	23.4	2314	1773	541	8.96	3.35	5.61	37	45	102.4
8 "	74	...	...	...	...	...	...	...	...	...	...	...
12 "	74	11.27	23.0	2302	1773	531	8.93	3.41	5.52	27	53	102.6
18 "	72	10.50	22.8	2296	1773	523	9.11	3.54	5.57	27	31	103.0
24 "	73	10.73	23.1	2305	1773	532	9.59	3.55	6.04	25	20	103.0
Goat 64. Male. Body weight 31.7 kg. Body surface 11,523 sq. cm. Percentage surface burnt 21.1. No pressure.												
Before burning	65	11.33	22.7	2207	1706	501	8.38	3.70	4.68	35	18	102.7
One hour after burning	78	12.83	25.9	2113	1566	547	8.00	3.31	4.69	35	57	103.4
2 hours "	85	13.50	26.7	2067	1516	551	7.78	3.11	4.67	40	71	103.4
4 "	88	13.59	27.5	2024	1468	556	7.25	3.17	4.08	40	74	102.1
6 "	85	12.72	28.1	1980	1424	556	6.45	2.98	3.47	43	18	101.5
8 "	80	...	...	...	...	...	...	...	...	...	...	...
12 "	80	12.12	27.7	2030	1468	562	6.53	2.96	3.57	46	27	102.0
18 "	74	10.61	25.7	2107	1566	541	6.70	3.05	3.71	49	19	102.7
24 "	75	11.46	25.9	2113	1566	547	7.22	3.08	4.14	45	18	102.3

TABLE III

*Weight of burnt limbs, burnt skin, lungs and heart, and the lung: heart ratio in treated and untreated goats*

Goat	Treatment	Percentage body surface burnt	Weight of total burnt limbs (g)	Weight of total burnt skin (g)	Weight (g) of		Lung heart ratio
					lungs	heart	
57	Pressure for 24 hours	20.0	1977 †	568	398	165	2.4
58		21.1	1990 *	658	352	132	2.6
63		20.9	2160 *	715	478	208	2.3
65		21.3	1905 *	545	452	142	3.2
66		20.6	2235 †	710	518	187	2.8
69		20.7	1800 †	495			
59	None	21.3	2475 *	950	355	134	2.6
61		20.7	2752 *	1065	494	184	2.7
62		23.6	2513 *	940	569	194	2.9
64		21.1	2250 *	905	378	172	2.2
67		21.0	2200 †	827	590	169	3.7
68		21.4	2205 *	835	357	145	2.5

\* Slight extension of oedema beyond burn margin † Moderate extension of oedema beyond burn margin

TABLE IV

*Recovery times and maximal rise or fall of haemoglobin, plasma volume and serum proteins in treated and untreated goats Percentage surface burnt given in recovery time column in brackets*

Blood component	Recovery time (hours)		Maximal rise or fall (per cent) *	
	Treated	Untreated	Treated	Untreated
Haemoglobin (per cent )	4 (21 1) 24 (20 0) 18 (20 9) 8 (21 3) 6 (20 0) 12 (20 7)	>24 (21 3) 24 (20 7) >24 (23 0) >24 (21 1) 24 (21 0) 24 (21 4)	16 18 13 8 9 7	17 33 45 35 33 36
Mean	(20 0)	(21 5)	11 83±1 87	33 17±3 71
Plasma volume (in c c )	6 12 >24 4 6 12 18 6	>24 24 >24 >24 >24 >24	14 15 8 5 6 17	32 38 32 17 33 36
Mean			10 83±2 09	31 33±3 03
Serum proteins (g per 100 c c )	18 24 >24 >24 18 24 24 6	>24 >24 >24 >24 >24 >24	12 12 13 11 13 4	16 27 17 23 19 18
Mean			10 83±1 39	20 0±1 71

\* Expressed as a percentage of the values found immediately before burning. The difference of the means of the maximal rise or fall is significant being 5, 5 and 4 times the standard error respectively.

Much the same conclusions were reached in series of animals with smaller burns. Of 6 goats with burns involving 7-10 per cent. total body surface, 3 were treated with pressure bandages, 3 serving as controls. Details of these experiments are not included here. Small groups of animals were also treated 4 and 6 hours after burning. Two of 4 goats in which a four-hours' delay occurred obtained benefit from pressure, but the course in 3 animals with pressure applied after 6 hours was not favourably influenced. Skin-tight pressure applied at once seemed useful in 2 of 3 goats, in which hæmoconcentration and plasma loss were reduced and the clinical condition was improved.

### DISCUSSION

Treatment of extensive limb burns with plaster bandages applied very soon after burning in such a fashion as to exert about 5-7 mm. Hg. pressure on the subcutaneous tissues has, we believe, given striking results. Plasma loss from the circulating blood is reduced, hæmoconcentration is decreased and local œdema appears lessened. It is necessary to apply the bandages with considerable overlap of burn margins, since pressure tends to squeeze some of the œdema fluid for some distance beyond the burn. Skin-tight bandages also give good results though they are not so effective as bandages applied with pressure. Delay in the application of pressure reduces the value of the method.

Our results support the favourable opinion expressed by an increasing number of observers, whose work is reviewed by Rossiter (1943; Rossiter and Peters, 1944) and Glenn, Gilbert and Drinker (1943). Evidence is also accumulating that pressure treatment encourages repair but we can express no views at this stage of our investigation, which is being continued to test this point. It is possible however to form some sort of idea about the physiological principles concerned in the initial stages. (1) Pressure leads to a reduction in burn œdema. Barnes and Trueta (1941) and Glenn *et al.* (1943) demonstrated this in dogs with closed plaster methods, Rossiter and Peters (1944) in rabbits and guinea-pigs with air cuffs. We have now shown that the same effect can be produced in goats by means of plaster bandages. (2) Pressure reduces fluid loss from the circulating blood, inhibiting hæmoconcentration and restricting the fall in serum protein concentration. Siler and Reid (1942), Lischer and Elman (1943), Sellers and Willard (1943) and Sellers and Goranson (1944) record less hæmoconcentration in dogs, we in goats. Siler and Reid found a reduction of protein loss but were not certain that this was statistically significant. Our results are more definite and we give evidence of reduced plasma loss based on direct measurements of plasma volume. (3) Pressure reduces the effects of increased capillary permeability in the burnt area. Glenn *et al.* found that less T. 1824 appeared in the lymph draining from plaster-coated burns than from untreated burns when this dye was administered intravenously. The

chemical changes in the blood which we present suggest an effect on permeability. (4) Pressure reduces lymph flow from a burnt area (Barnes and Trueta ; Glenn *et al.*). (5) The application of a pressure bandage raises the subcutaneous tissue pressure as we show in this report.

These facts can be explained most simply by the assumption that pressure combats the altered capillary permeability typical of a burn through increasing the tissue pressure. Local plasma loss is reduced, plasma volume decrease is inhibited and the main danger during the first stages after burning is lessened.

Finally we wish to stress several observations which have come out of this investigation.

1. Loss of plasma after burning seems to be local only, the amount of local oedema on the whole agreeing well with the amount of fluid lost from the blood. We found no sign of effusion into the serous sacs and no pulmonary oedema or other form of fluid loss. A slight but widely diffused plasma loss would, however, be difficult to discover.

2. Attempted compensation for plasma loss can be demonstrated at a very early stage after burning. When 3 goats were burned to the extent of 22 per cent. of the total body surface, the affected skin and subcutaneous tissue half-an-hour later weighed 617 g. on an average. The same amount of unburnt skin from goats of similar sex, age and body weight averaged 322 g. The burnt areas had thus increased 295 g. on an average in 30 minutes, due partly to plasma infiltration and partly to hyperæmia, although the latter factor is probably small. At this time the mean decrease in plasma volume in the burnt goats was 118 g., estimated by the dye method. In other words, though the burnt areas had gained an average of 295 g. fluid in 30 minutes, the blood plasma volume had fallen only 118 g. It seems that fluid must have been added to the circulating fluid through some compensating mechanism.

3. Pathological changes in the organs have been slight, even though our animals were near the lethal border-line. Mild liver damage occurred in only one goat. We conclude that death from burning during the first 24 hours is most likely circulatory in origin.

4. Burnt goats are susceptible to certain coincidental conditions, especially struggling, fear, pregnancy and probably anæmia. Cirrhosis of the liver, present in 4 of our animals, was without effect.

#### SUMMARY

1. Goats exposed to extensive thermal burning (about 20 per cent. total body surface) rapidly lose blood plasma into the burnt area, amounting at times to as much as 40 per cent. of the total plasma volume. This is accompanied by hæmoconcentration, slight transient hæmolysis, slight increase in blood non-protein nitrogen, decreased plasma and total blood volume and a steady decline in the serum protein concentration.



2. Pathological changes are slight during the first 24 hours after burning. Hæmolysed blood is excreted by the kidneys in small amounts. Liver damage is insignificant.

3. Plaster pressure bandages applied to extensive burns of the extremities reduce hæmoconcentration, loss of fluid and serum protein from the circulation and local œdema. The clinical course appears to be favourably affected.

4. Delay in pressure bandaging diminishes the value of the treatment. Skin-tight bandages give useful results, though inferior to those obtained when pressure is applied.

5. The physiological principles of pressure treatment are discussed and the opinion is advanced that pressure combats increased capillary permeability in a burnt region.

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BACTERIOLOGICAL ASPECTS OF PENICILLIN  
THERAPY \*

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THE evaluation of a new chemotherapeutic drug is always a matter of some difficulty and particularly so when supplies are restricted. Ehrlich's brilliant researches in this field have enabled us to remove much of the empiricism in which chemotherapy was shrouded. Nowhere can this be better exemplified than in the case of penicillin, where the laboratory is able to give accurate information as to the sensitivity or insensitivity of the infecting micro-organism to the drug as well as to the extent to which the drug has penetrated into the blood and tissues after injection. During the past two years a research on the clinical application of penicillin at the Middlesex Hospital has given us an opportunity of studying this subject from the bacteriological aspect, of which we give an account below.

## METHODS

*Estimation of penicillin sensitivity of bacteria*

We have based our estimations on the Oxford unit of penicillin, defined by Florey and Jennings (1942, p. 122) as "that amount of penicillin which when dissolved in 50 ml. of meat extract broth just inhibits completely the growth of the test strain of *Staphylococcus aureus*". An example of a test of penicillin sensitivity which consists of incubating the organism overnight in 1 c.c. quantities of serial two-fold dilutions of calcium penicillin in peptone broth in 3" x  $\frac{1}{2}$ " tubes is shown in table I. For fastidious bacteria the most usual enrichment has been 2 per cent. glucose, sometimes with the addition of 5 per cent. serum. In the case of actinomyces we have used peptone broth with the addition of 2 per cent. glucose, 0.2 per cent. agar and a small quantity of iron filings, the result being read after 48 hours' aerobic incubation.

*Estimation of penicillin content of serum and other fluids*

For the estimation of penicillin by this method 1 c.c. of serum or other fluid is required. If the fluid cannot be examined immediately, it should be stored at refrigerator temperature. Turbid fluids, as from empyemata or abscesses, should be partially clarified by centrifuging.

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\* This work was done on behalf of the Penicillin Research Committee of the Middlesex Hospital under the auspices of the Medical Research Council.

This test is similar to that for penicillin sensitivity except that the unknown quantity is now the concentration of penicillin and the Oxford staphylococcus

TABLE I

*Example of test for penicillin sensitivity*

Organism	Penicillin concentration in units per 50 c.c.								Control
	16	8	4	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	
Oxford staphylococcus . . .	—	—	—	—	—	+	+	+	+
<i>Staphylococcus aureus</i> I . . .	—	+	+	+	+	+	+	+	+
<i>Staphylococcus aureus</i> II . . .	—	—	—	—	—	—	—	+	+

Result :—*Staphylococcus* I is 16 times more resistant to penicillin than the Oxford staphylococcus.

*Staphylococcus* II is 4 times more sensitive to penicillin than the Oxford staphylococcus.

is used as a standard (table II). If there is any question of contamination in the fluid to be tested, as in pus, this method requires modification so that the test organism can be easily distinguished in reading the result. This is done

TABLE II

*Test for penicillin content of serum*

Dilution of serum . . . . .	1:1	1:2	1:4	1:8	1:16	1:32	Control
Growth of Oxford staphylococcus at 18 hours	—	—	—	—	+	+	+

Result :—Serum inhibits growth of the Oxford staphylococcus in a dilution of 1:8.

by inoculating the tubes with one drop of a 1:500,000 dilution of an over-night culture of the test organism, adding 0.5 c.c. of 2 per cent. nutrient agar to each tube and sloping before incubation. The test organisms then appear as numerous discrete colonies which are readily distinguished from any growth arising from bacteria already present in the fluid for assay.

#### *Cultivation of bacteria from fluids containing high concentrations of penicillin*

We have frequently obtained cultures of penicillin-sensitive bacteria by straightforward plating from abscess fluids containing high concentrations of penicillin, as is shown in fig. 2. We have also obtained profuse growths of staphylococci from breast abscesses in which the abscess fluid was bacteriostatic in dilutions of 1:1000, 1:4000 and 1:8000. The presence of penicillin, however, was demonstrated by a zone of inhibition where the inoculum first touched the plate. Our experience would therefore indicate that in these fluids the amount of penicillin present is not as a rule sufficient to necessitate any special measure to destroy it, such as the use of penicillinase (Abraham and Chain, 1940; Harper, 1943; Duthie, 1944).

## RESULTS

*Penicillin sensitivity tests*

*Staphylococcus aureus.* The sensitivity of 157 coagulase-positive strains of *Staphylococcus aureus* is shown in the form of a distribution curve in fig. 1. The peak of the curve corresponds to 79 strains of staphylococci that were equal in sensitivity to the Oxford staphylococcus, but it will be noted that the curve is skew, since most of the remaining staphylococci were more resistant. In fact, only one strain was as much as 8 times more sensitive than the

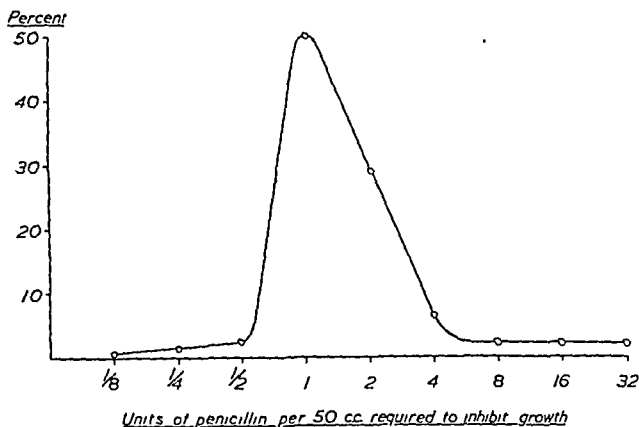


FIG. 1.—Distribution curve of the penicillin sensitivity of 157 coagulase positive strains of *Staphylococcus aureus*. The peak of the curve corresponds to 50 per cent. of the strains which are equal in sensitivity to the Oxford staphylococcus, being completely inhibited by a concentration of 1 unit of penicillin in 50 c c. of peptone broth.

Oxford staphylococcus, whereas four were 64 times, and one more than 500 times more resistant.

These tests on *Staphylococcus aureus* have afforded a useful means of gauging the sensitivity of other types of bacteria, as is shown in table III. So far we have regarded those bacteria which come within the range of 4 times more or 4 times less sensitive than the Oxford staphylococcus as being of average sensitivity and capable of responding normally to penicillin treatment. On this basis 90 per cent. of the strains of *Staphylococcus aureus* were of average sensitivity, only one was highly sensitive, and 9 per cent. were relatively resistant.

*Streptococci and other organisms.* Most of the 33  $\beta$ -haemolytic strains were 2-4 times more sensitive than the Oxford staphylococcus

and 7 were highly sensitive; the non-hæmolytic and *viridans* strains were on the whole more resistant. The 7 strains of pneumococci tested, including strains of types III and VII, were all sensitive, whereas of 8 diphtheroid organisms 4 were resistant.

TABLE III

*Penicillin sensitivities of bacteria isolated from cases proposed for penicillin treatment*

Organism	No. of strains tested	Highly sensitive	Sensitive	Less sensitive
<i>Staphylococcus aureus</i> .	157	1	141	15
<i>Staphylococcus albus</i> .	14	0	10	4
<i>Streptococcus pyogenes</i> .	33	7	25	1
<i>Streptococcus viridans</i> .	6	0	4	2
Non-hæmolytic streptococci	17	4	7	6
Pneumococcus .	7	0	7	0
Diphtheroid organisms .	8	0	4	4
Actinomyces, human strains	10	0	6	4
Actinomyces, bovine strains	3	0	0	3

Highly sensitive = more than 4 times more sensitive than the Oxford staphylococcus.

Sensitive = from 4 times more sensitive to 4 times less sensitive than the Oxford staphylococcus.

Less sensitive = more than 4 times less sensitive than the Oxford staphylococcus.

**Actinomyces.** We have also included the results of our sensitivity tests on 10 strains of actinomyces isolated from human cases of actinomycosis. These human strains can be differentiated by their cultural characters and fermentation reactions into two groups. These groups also differ in their sensitivity to penicillin, one being 2-4 times more sensitive than the other but all have been within a range capable of being controlled by penicillin therapy. We have also tested 3 old laboratory strains originally derived from cattle infections. Their relationship to the human types is not yet quite clear but they all appear to be much more resistant.

### *Penicillin estimations*

#### Penicillin content of blood serum

*After intramuscular injection.* A number of estimations were made on the blood of patients undergoing treatment by 3-hourly intramuscular injections. Most of these were made  $\frac{1}{4}$  hour and  $2\frac{3}{4}$  hours after the injection of 10,000 or 20,000 units in adult subjects: the results are shown in table IV. As would be expected the bacteriostatic dilution of the serum is in general much higher a quarter of an hour after injections of 20,000 units than after injections of 10,000 units. On the other hand, the blood levels at the end of  $2\frac{3}{4}$  hours are much the same with both doses. It would thus appear that the bacteriostatic level falls off much more rapidly with the larger dose. How rapid this

fall can be has been shown in one case where the standard bacteriostatic dilution was 1 : 32 at 20 minutes and fell to 1 : 4 in the next 10 minutes.

TABLE IV  
*Inhibitory power of sera expressed as dilutions after intramuscular injections of penicillin*

Interval after injection	Penicillin dose (units)	Number of samples having inhibitory powers of				
		1:64-1:16	1:8-1:4	1:2	1:1	<1:1
$\frac{1}{2}$ hr.	20,000	11	4	0	0	0
	10,000	2	4	1	0	0
2 $\frac{1}{2}$ hrs.	20,000	0	3	3	8	2
	10,000	0	1	2	4	2

It is to be noted, however, that the bacteriostatic level of the undiluted serum fell short of complete inhibition of the Oxford staphylococcus in only 4 out of the 25 samples taken at the end of the 3-hour injection period. It may therefore be concluded that this method of administration maintains a bacteriostatic level in the blood throughout treatment besides giving high concentrations for a period after each injection.

*During administration by intramuscular drip.* Repeated estimations have also been made of the penicillin content of the sera of patients during the administration of penicillin by intramuscular drip. The dosage rates per 24 hours were 240,000 units in one patient and 120,000 units in five. As is shown in table V a standard bacteriostatic

TABLE V  
*Inhibitory power of sera expressed as dilutions during administration by intramuscular drip*

Penicillin dose in 24 hrs. (units)	Number of samples having inhibitory powers of			
	1:8-1:4	1:2	1:1	<1:1
240,000	2	3	2	0
120,000	1	13	7	1

titre of 1 : 2 was exceeded in only 3 of 29 samples. The dosage levels used here correspond to 15,000 and 30,000 units every 3 hours and are therefore of the same order as those in table IV. A comparison of the results shows that the intramuscular drip method maintains a slightly higher bacteriostatic level than that usually present 3 hours after a single intramuscular injection at the same dosage rate, but it has the disadvantage of not providing the higher, although temporary, penicillin levels characteristic of intermittent intramuscular injection.

## Penicillin content of empyemata

The standard bacteriostatic dilutions of empyema fluids made during the administration of 20,000 units of penicillin intramuscularly every 3 hours ranged from 1 : 2 to 1 : 8, showing that some penetration of penicillin was obtained.

In 5 cases of empyema where treatment was controlled by estimations of the penicillin content of the aspirated fluid at various intervals after injections of penicillin directly into the infected cavity, a tendency towards increasing absorption was noted during resolution. The findings in a case of chronic empyema infected with *Staphylococcus aureus* are shown in fig. 2. In the first place it will be noted that the

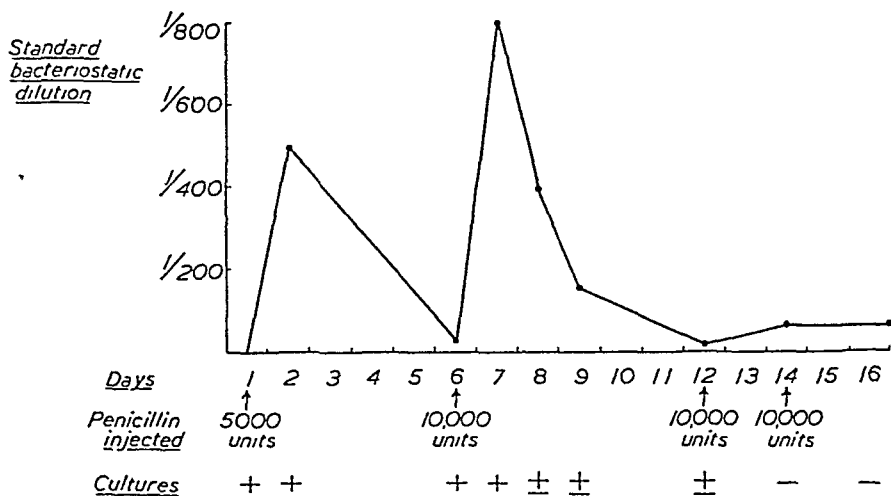


FIG. 2.—Penicillin content and disappearance of *Staphylococcus aureus* in an empyema after repeated aspiration and injection of penicillin.

presence of penicillin can be detected up to 5 days after it has been injected, so that penicillin would appear to be reasonably stable under these conditions. It is also to be noted that the penicillin content of the fluid fell off much more rapidly after the later injections and that this increase in the absorption of penicillin was associated with a gradual diminution and final disappearance of the infecting organism. The increasing rate of absorption of the penicillin would therefore appear to be due to the clearing up of the infection which would provide new channels for absorption.

The influence of the state of the pleural lining was also shown in a case of post-influenzal streptococcal bronchopneumonia with an acute pleural effusion in which 500 c.c. of slightly turbid fluid was aspirated and replaced by 20,000 units of calcium penicillin in 50 c.c. of saline. In this acute case the penicillin was so rapidly absorbed that two days later its presence could not be detected even in the undiluted fluid.

### Penicillin content of pus from abscesses

Estimations were made of the penicillin content of the pus from abscesses which were treated by repeated aspiration and the injection of calcium penicillin into the infected cavity. In abscesses which had developed in association with osteomyelitic lesions the replacement of 10-15 c.c. of pus by 1-4 c.c. of saline containing 1000 units of penicillin was followed 24 hours later by standard bacteriostatic dilutions of the fluid varying from 1 : 32 to 1 : 256, showing that the dosage of penicillin was adequate. In breast abscesses injections of 10,000-20,000 units of penicillin in 10-15 c.c. of saline on alternate days were followed 2 days later by bacteriostatic dilutions varying from 1 : 16 to 1 : 130,000. The variability of the rate of absorption of penicillin from these abscesses would appear to be due largely to the state of the wall of the abscess, as has already been discussed in relation to empyema.

### Penicillin content of cerebrospinal fluid

It has already been shown by Fleming (1943) that there is little interchange of penicillin between the blood-stream and the cerebrospinal fluid. We have confirmed this on a diagnostic lumbar puncture fluid which had a bacteriostatic dilution 16 times less than the blood serum 30 minutes after an intramuscular injection of 20,000 units of penicillin. We were, however, able to obtain some evidence that the disappearance of penicillin from the cerebrospinal fluid is altered during infection. In 2 cases of pneumococcal meningitis which were treated with daily intrathecal injections of 5000 and 10,000 units of penicillin respectively, the fluid, at first turbid, cleared up in 2 to 3 days. Twenty-four hours after the first 2 daily injections, penicillin could be detected in the cerebrospinal fluid only by the use of a highly sensitive test organism, but after the third injection the standard bacteriostatic dilutions of the cerebrospinal fluids were 1 : 16 and 1 : 64 after doses of 5000 and 10,000 units respectively, showing that the amount of penicillin retained after 24 hours had increased by 32 and 16 times. This increased retention is most probably related to resolution, leading to restoration of the normal barrier between the blood and the cerebrospinal fluid.

### *Effect of treatment on infecting organisms*

*Systemic treatment.* The effect of penicillin on the infecting organism in a number of cases is briefly summarised in table VI, which includes only completed cases where it was possible to obtain repeated specimens for examination. There is considerable variation in the rate of disappearance of the infecting organisms, which tend to persist longer in devitalised and necrotic tissue associated with



carbuncles and bone infections, and in the sputum of pulmonary cases. In some cases, especially lung infections, these persistent organisms appear to be saprophytic and have no unfavourable influence on the recovery of the patient, as is indicated in table VI, where it is

TABLE VI  
*Bacteriological findings in 32 treated cases*

Infecting organism and type of infection	Number of cases	Disappearance of infecting organisms			Treatment failed	Secondary infections
		within 7 days	in 7-14 days	after 14 days		
<i>Staph. aureus</i>						
Osteomyelitis . . .	8	1	2	5	4	5
Bronchopneumonia . .	3	1	0	2	0	0
Soft tissue infections .	12	7	2	3	1	5
<i>Strep. pyogenes</i>						
Osteomyelitis . . .	2	2	0	0	0	0
Bronchopneumonia . .	2	1	0	1	1	0
Superficial infections .	1	1	0	0	0	0
<i>Strep. viridans</i>						
Osteomyelitis . . .	2	0	0	2	2	1
<i>Staph. aureus</i> and <i>Strep. pyogenes</i>						
Superficial infections .	2	1	1	0	0	1
Totals .	32	14	5	13	8	12

shown that in 5 cases treatment succeeded in spite of the persistence of the infecting organism. The persistence of organisms in a case which is apparently doing well should not therefore be always regarded as a discouraging sign in penicillin therapy.

The 12 secondary infections noted in table VI can be attributed to the fact that many of the lesions were of the type in which secondary infections occur with great readiness, even when stringent precautions are taken. These secondary infections, however, were all superficial, being confined to surface lesions or occurring in relation to drainage tubes, and all were successfully treated by appropriate antiseptic measures.

*Development of resistant organisms during systemic treatment.* An increase in the resistance of the infecting organism to penicillin was detected in only 5 cases. In 2 cases of staphylococcal osteomyelitis and in one of  $\beta$ -haemolytic streptococcal empyema the infecting organisms withstood 4 times more penicillin at intervals of 6, 11 and 20 days respectively after treatment commenced. The organisms however were still within the range of average sensitivity and their increased resistance did not appear to have any unfavourable influence on the recovery of the patient. In the 4th case, one of staphylococcal endocarditis with a fatal outcome, an 8-fold increase in resistance was

found on the 16th day of treatment. In the 5th case, a staphylococcal cavernous sinus thrombosis, a 4-fold increase in resistance was detected following a course at the usual level of 20,000 units per 3 hours. The patient was then put on double dosage with good clinical result, but a further examination of the organisms a week later showed a 16-fold increase. From this case it is evident that if the penicillin content of the blood can be raised sufficiently, infections by relatively resistant organisms can be overcome.

### *Penicillin ointment*

A few early cases were treated locally with a penicillin ointment consisting of a lanette wax base containing 250 units of penicillin per gramme. It was, however, suspected that the results were not commensurate with the amount of penicillin used and investigation showed that only a small proportion of penicillin could be expected to diffuse into the infected area. Thus in a series of test-tube experiments designed to show how much penicillin would diffuse from the ointment into water or plasma at body temperature, it was found that only a minimal amount diffused out during the first few minutes and none thereafter. Further experiments with proflavine or sulphonamides in this ointment base gave similar results and it became apparent that continuous diffusion of a solute from such an emulsion could not be readily obtained. The use of penicillin ointment was therefore discontinued at an early stage and since then penicillin has been applied only as a solution or as a powder diluted in a sulphonamide to give a concentration of 5000 units per gramme.

### DISCUSSION

Our sensitivity tests on *Staphylococcus aureus* have shown that 90 per cent. of the strains met with in these investigations were of a sensitivity within the range of 4 times more and 4 times less than the Oxford staphylococcus. Only 9 per cent. were relatively resistant and probably would not have responded to treatment with small doses of penicillin. Variations in sensitivity were also found in other organisms, and it is evident that there is a certain proportion of comparatively resistant strains in those groups of bacteria which are generally sensitive to penicillin. This fact must be borne in mind in the selection of cases for treatment and in assessing dosage. If the infecting organism in a particular case is highly resistant, it is probable that penicillin treatment would be ineffectual, but if the degree of resistance is only moderate the patient would probably respond to a dosage of penicillin higher than that usually administered.

An increase in the resistance of the infecting organism to the action of penicillin was detected in only 5 cases. The degree of resistance developed was sufficient to allow the organism to with-

stand concentrations of penicillin up to 16 times greater than at the commencement of treatment but this development of resistance can usually be overcome by adequate dosage. The ease with which resistance to penicillin can be induced in bacteria has already been demonstrated *in vitro* by Abraham *et al.* (1941), Rammelkamp and Maxon (1942) and McIntosh and Selbie (1943), and indicates the necessity of employing maximal doses at the outset to produce an immediate result.

The most efficient method of administering penicillin is still a matter for discussion and apparently is dependent mainly on its rapid elimination by the kidneys. The failure to get a high blood titre with the intramuscular drip is a problem which is not fully understood and may be dependent on a number of factors, some of which we may be able to overcome. One unfavourable factor which can be readily avoided is the destructive action on penicillin of some samples of rubber tubing which, in our experience, may cause a 16-fold drop in the activity of penicillin solutions in 24 hours. However, where a high blood content is required it is necessary to give the penicillin in large doses by intramuscular injections. We admit that these high levels are only temporary, but it would appear that they are capable of (a) penetrating into closed cavities, and (b) overcoming resistant organisms as in the case where a 16-fold increase in resistance occurred during treatment.

Absorption of injected penicillin from abscesses or empyemata depends mainly on the state of the wall of the cavity. Where there is a thick wall of granulation tissue the penicillin is not rapidly absorbed, while in an acute serous effusion it disappears with great rapidity. With resolution of the more chronic conditions penicillin disappears much more rapidly. On the other hand, absorption of penicillin from the cerebrospinal fluid is greatly increased by the presence of an inflammatory process in the meninges.

A completely satisfactory vehicle for the local administration of penicillin is a pressing need, as it would appear that the release of penicillin from oily or fatty vehicles is difficult, making this an uneconomical method of use.

It has been noted in several cases undergoing treatment that a rapid amelioration in the patient's condition with a fall in the leucocyte count does not coincide with the disappearance of the bacteria. There is no dramatic disappearance of the infecting organism as in the arsenical treatment of syphilis, but in some way penicillin affects the host-parasite relationship to the great benefit of the patient, as though it had rendered the bacteria avirulent and allowed full play to the natural defences of the body. We have been investigating this mechanism but are unable to demonstrate that penicillin has any direct stimulating effect on the formation of antibodies.

We have frequently isolated bacteria from the neighbourhood of the lesion at considerable intervals after apparent cure. These may

represent a recrudescence of the primary organism or perhaps a re-infection by another strain. This type of recurrence is mainly noticeable with staphylococci, but until staphylococci can be typed we are unable to determine their origin.

In view of the fact that penicillin is ineffective against *Ps. pyocyanea*, *Proteus* and the coliform organisms, secondary infections by the Gram-negative bacilli have to be guarded against by careful technique in the treatment of open wounds. Infection in these cases, however, was mild and superficial and usually responded readily to wound antiseptics, especially sulphathiazole-proflavine powder (McIntosh *et al.*, 1944).

### SUMMARY

A series of infections specially selected for penicillin treatment has been investigated from the bacteriological aspect.

*Staphylococcus aureus* is in the great majority of cases sensitive to penicillin but considerable variation was shown in the degree of sensitivity: 15 out of 157 strains tested were at least 8 times less sensitive than the standard strain. *Streptococcus pyogenes* and pneumococci were usually sensitive and showed only minor degrees of variation in susceptibility. The non-haemolytic streptococci were of varying sensitivity, some showing considerable resistance. The 10 strains of actinomyces tested were somewhat less sensitive than the Oxford staphylococcus but the majority were within the therapeutic range of penicillin.

Observations on penicillin blood levels during therapy would indicate that a dosage of 20,000 units given intramuscularly every 3 hours will maintain a bacteriostatic concentration in the blood and tissues sufficient to deal with all types of sensitive organisms likely to be encountered.

The penetration of penicillin from the blood into cavities and in particular the subarachnoid spaces (cerebrospinal fluid) is at times insufficient and has to be supplemented by direct injection.

A good indication of improvement in these pyogenic infections is a diminution in the blood leucocyte count.

The rate of disappearance of the micro-organisms depends largely on the nature of the lesion. In simple acute infections they disappear as a rule in a few days but persist longer in deep foci and in areas of devitalised or necrotic tissue. This persistence is especially characteristic of lung infections, where they appear to be living a purely saprophytic existence.

Secondary invasion by penicillin-insensitive organisms is liable to occur, especially in severe wounds sustained in battle or air-raids. The necessity of vigorous counter-measures against such micro-organisms cannot be too strongly emphasised.

The development of resistance to the drug by the infecting micro-organism during treatment was observed in a small number of instances.

Our observations confirm the outstanding value of penicillin as an antibacterial chemotherapeutic agent.

We wish to thank Dr R. H. M. Robinson for his help in the bacteriological control of cases treated in the Sector.

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## THE IN-VITRO DETERMINATION OF THE SULPHONAMIDE SENSITIVITY OF BACTERIA

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(PLATE XII)

It is often desirable to determine whether an organism responsible for an infection is sensitive or resistant to sulphonamides. The methods commonly in use for this purpose are: (1) the ditch plate (Fleming, 1940-41; Colebrook and Francis, 1941), (2) the streak plate (Medical Research Council War Memo. no. 10, 1943), (3) tests carried out in broth, serum broth, and peptone water (Lowell, Straus and Finland, 1940-41), (4) tests carried out in blood, with subcultivation to determine the end-point, (5) tests carried out in blood, using hæmolysis as the indicator of growth. In our experience each of these methods has disadvantages which are noted below.

1. *The ditch plate.* (a) Although this method offers a striking demonstration of the difference between sensitive and resistant strains, it does not serve so well as a quantitative test because the sulphonamide concentration acting on the bacteria is unknown and is, in fact, constantly changing as the drug diffuses out of the ditch. Chemical estimations of the sulphonamide in strips of agar cut out from such plates at intervals after preparation have been made by Fuller and by Bowers (personal communications to Dr Colebrook), and also by Miss Semeonoff working in this laboratory. They show (fig. 1) that the sulphonamide concentration in the ditch falls rapidly during the first 24 hours and then more slowly, simultaneously the concentration in the peripheral parts of the plate rises. In consequence of this changing gradient it happens that bacteria which are just inhibited at, say, 2 cm. from the ditch during the first 24 hours' incubation are exposed to a lower concentration of sulphonamide on the second day and may grow sufficiently to form colonies nearer to the ditch. In such cases the ditch test does not give a clear index of sensitivity. Examples of this are shown in table I (strains 1, 2 and 3). This table gives further evidence of the change in sulphonamide concentration. When the other side of the same plate was seeded with the same strains at 24 hours, i.e. when the sulphanilamide concentration in the ditch had fallen and that in the peripheral parts of the plate had risen, the more resistant strains (1 and 2) grew right up to the ditch, the more sensitive strains (4 and 6) showed an inhibitory zone wider than with the initial seeding.

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\* Both working for the Medical Research Council.

shown by Fuller, Colebrook and Maxted (1939), and this probably explains our findings with different bloods.

### *Investigation*

The object of this investigation was to find a method suitable for routine use which fulfilled the following conditions: (1) the results obtained to be clear-cut, (2) the sulphonamide concentration to which the bacteria are exposed to be known and constant, (3) the technique to be simple and rapid, (4) the method to be applicable to most of the common pathogens.

A solid medium containing a known concentration of sulphonamide and taking the presence or absence of growth from small inocula as the criterion of sensitivity seemed likely to meet these requirements if a medium free from sulphonamide-antagonising substances could be found. Colebrook and Francis recognised that this condition was essential for the ditch plate method and recommended the selection of suitable batches of agar by trial and error, using known sensitive and resistant strains as indicators. All the nutrient media we have tested contained sulphonamide-antagonising substances, which were neutralised after adding horse blood and incubating the mixture for at least a few hours. The necessity for the period of incubation was overlooked by Colebrook and Francis owing to the fact that they set aside plates prepared for ditch testing (usually overnight) to allow diffusion of sulphonamide from the ditch.

The presence of sulphonamide antagonist in nutrient agar and its neutralisation by horse blood were demonstrated by the following experiment. An agar plate containing 5 mg. per 100 c.c. sulphathiazole and 5 per cent. horse blood was poured, dried for 30 minutes at 37° C. and immediately inoculated with known sensitive and resistant strains of *Staph. aureus*. Next day the same strains were inoculated on the other half of the same plate, which was then reincubated. The strains planted immediately after pouring all grew well in spite of the sulphathiazole present; of those planted 24 hours after pouring only the resistant strains grew. When the experiment was repeated with a plate made from the same agar, but containing no horse blood, the sulphathiazole failed to inhibit the sensitive strains even after keeping the plate for 24 hours at 37° C. (fig. 2). These results indicate that some factor present in horse blood neutralises the sulphonamide-antagonising substances present in nutrient agar and that the action is relatively slow. The neutralisation is more rapid at incubator temperature than at room temperature or in the refrigerator. The mechanism of the process remains obscure but further investigation has brought to light the following facts. (1) Horse blood is the only blood tested which has shown neutralising powers; human, rabbit and sheep bloods have none. (2) The neutralising factor of horse blood is confined to the red cells and acts more readily after lysis of the cells. Fresh whole horse blood (less

## SULPHONAMIDE SENSITIVITY OF BACTERIA

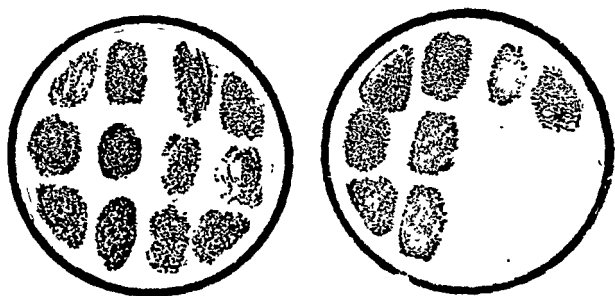


FIG. 2.—Neutralisation of sulphonamide antagonist in agar by incubation of medium in presence of horse blood.

*Left* Nutrient agar plate containing 5 mg. per 100 c.c. of sulphathiazole.

*Right* Nutrient agar plate containing 5 mg. per 100 c.c. of sulphathiazole plus 5 per cent. of horse blood

Left half of each plate inoculated with 6 strains of *Staph. aureus* immediately after pouring, right half inoculated after 24 hours' incubation at 37° C. The top two strains on each half of the plates are sulphathiazole-resistant controls.

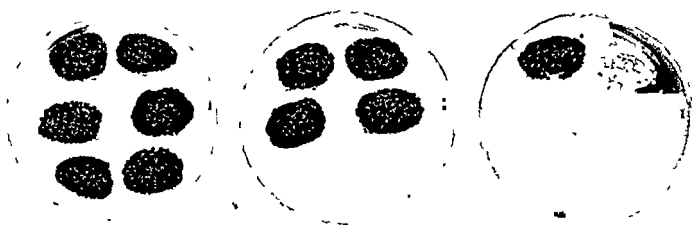


FIG. 3—Routine sensitivity test with *Staph. aureus* (6 strains) and sulphathiazole

*Left* Control plate containing no drug.

*Centre*: Plate containing 1 mg. per 100 c.c. sulphathiazole.

*Right*: " " " " " "

The upper strains on each plate were resistant, the middle strains partially resistant, the lower strains sensitive,





than 4 days old) neutralises the antagonist only after lysis; old blood, kept in the refrigerator for 2-3 weeks, which has already undergone some degree of hæmolysis and in which the intact red cells are doubtless more easily lysed, works well. (3) Both defibrinated and oxalated blood are suitable.

Twenty batches of agar (12 of meat infusion, 5 of meat digest, 2 of lemco and 1 of casein-yeast agar) and 3 of nutrient broth, prepared in 9 different laboratories, and 4 samples of peptone, were tested for the presence of sulphonamide antagonist. It was found in all. The amount present in each of a number of batches of agar medium was determined by incorporating varying quantities of lysed horse red cell solution in sulphonamide plates prepared from it and noting the smallest concentration that allowed growth inhibition of a sensitive bacterium. The results are shown in table II along with

TABLE II

*Concentrations of lysed horse red cell solution necessary to neutralise sulphonamide (S) antagonist in nutrient agar and peptone water containing 10 mg. sulphanilamide per 100 c.c.*

Batch no.	Medium	Percentage of lysed horse red cell solution required for neutralisation of S antagonist
1	Meat digest agar	2.0
2	" " "	2.0
3	" " "	1.0
4	Meat infusion agar	0.5
5	Casein-yeast agar	1.0
6	Lemco agar	2.0
7	Commercial peptone I	2.0
8	" " II	0.5-1.0
9	" " III	4.0
10	" " IV	4.0

those of the 4 samples of peptone examined. With these nutrient media 4 per cent. or less of lysed horse blood was sufficient to neutralise the antagonist; with 3 batches of agar, not shown in table II, it proved more difficult to neutralise.

The antagonist could be removed from nutrient broth by adding the appropriate amount of lysed horse cell solution (usually 5 per cent., but determined by titration with the medium used), incubating the mixture overnight at 37° C. and coagulating the blood proteins by steaming for 30 minutes; the resulting precipitate was removed by filtration. For routine testing it is not necessary to clear media of antagonist in this manner; the addition of lysed blood at the time of preparing the plates or slopes effectively neutralises the antagonising activity of the medium. That the antagonist present in nutrient agar is not derived from the agar itself was shown by rendering nutrient broth antagonist-free by the above method and preparing

solid media from it by the addition of agar. Plates so prepared showed no antsulphonamide activity.

That the sulphonamide antagonist present in culture media was not *p*-aminobenzoic acid was shown by Macleod (1940); and we found that *p*-aminobenzoic acid was just as active in the presence of lysed horse red cells as in an antagonist-free medium.

The 3 batches of nutrient agar referred to above, in which the antagonist was not readily neutralised, were further investigated. Two of these agars (prepared in the same laboratory) were mixed with 25 per cent. lysed cell solution and after 3 days' incubation the blood proteins were removed. Some antsulphonamide activity was still present but was effectively neutralised by the addition of a further 5 per cent. of lysed cell solution at the time the plates were poured. The third batch of agar was more easily cleared of antagonist; the addition of 10 per cent. lysed blood and 2 days' incubation before removing the blood proteins rendered it suitable for sulphonamide sensitivity tests. This indicates that these batches of agar contained either much more antagonist than was found in the other samples tested or another type of antagonist which was more difficult to neutralise.

#### *Suggested routine procedure for sulphonamide sensitivity tests*

The method finally adopted was the comparison of growth of the strain to be tested on a series of plates or slopes containing known concentrations of the drug. It is more laborious than the ditch plate method, but there is no other simple method of avoiding the fallacies of the ditch plate. The medium used is first cleared of sulphonamide antagonists by treatment with lysed horse blood.

*Control strains.* For the titration of media and for controlling routine tests it is necessary to have strains of known sensitive, resistant and partially resistant bacteria.

*Preparation of lysed horse red cell solution.* The solution is prepared by replacing the plasma or serum from oxalated or defibrinated horse blood with sterile water and freezing solid overnight. If complete lysis is not achieved the blood can be returned to the freezing chamber for a further 24 hours. The solution appears to keep indefinitely in the refrigerator.

*Titration of agar.* To 10-ml. quantities of melted agar containing 10 mg. per 100 c.c. of sulphanilamide increasing quantities (0.25-8 per cent.) of lysed cell solution are added; plates are poured, dried off for 30 minutes and inoculated with the control strains of known sensitivity. The lowest concentration of lysed cell solution which allows the sulphanilamide to inhibit the growth of the sensitive and partially resistant strains, while the fully resistant strains grow, is the amount needed to make the agar suitable for immediate use. With most agars which we have tested the concentration required was

less than 5 per cent., but it is advisable to have at least this concentration present in plates used for routine testing to ensure a good growth of fastidious organisms.

*Concentrations of sulphonamide.* The concentrations we have used for testing the pyogenic cocci cover the range most commonly found in the blood stream during sulphonamide therapy, namely 2 and 10 mg. per 100 c.c. of sulphanilamide and 1 and 5 mg. per 100 c.c. of sulphathiazole. Strains which are inhibited by both concentrations are rated as sensitive, those showing growth only in the presence of the lower concentration as partially resistant and those growing on all plates as resistant.

*Preparation of plates and slopes.* These are easily poured from stock bottles of sulphonamide agar. The sulphonamide agar is best prepared by adding the appropriate amount of a strong aqueous solution of the drug to the melted agar. The practice of adding the dry powder to the agar is not recommended, as it is difficult to ensure complete solution and even distribution. To the sulphonamide agar so prepared the lysed cell solution is added, mixed well and the plates poured. In laboratories where the testing of cultures for sensitivity is only carried out occasionally the use of plates is wasteful and the testing can be carried out satisfactorily on slopes prepared in  $\frac{1}{4}$  oz. screw-capped bottles. These slopes can be stored in the refrigerator for long periods.

*Cultures for testing.* The organism under test is grown overnight in an appropriate fluid medium—broth, serum broth or peptone water—and diluted 1:1000 in 10 per cent. broth-saline. One 2-mm. loopful of this diluted culture is rubbed over a section of the plate or on a slope. Control plates or slopes containing no drug should be included in all tests to ensure that growth takes place from the inoculum used (fig. 3).

This method has been used for the testing of the pyogenic cocci, a few strains of pneumococci, coliform organisms and *Clostridia*\* with various sulphonamides and has given clear-cut, easily read results. Complete agreement was obtained when 22 strains of *Strep. pyogenes* and 26 strains of *Staph. aureus* were tested by the lysed blood plate and by cultivation in heated human blood plus sulphonamide, with subsequent explanting.

The advantages of the method described are as follows. (1) The technique is simple and the results obtained are clear-cut. (2) The concentration of the drug acting on the bacteria is known and constant. (3) The plates and slopes can be kept ready for use. (4) The method is suitable for a wide range of organisms. (5) Results are obtained in 24 hours. (6) The agar plate technique is suitable for determining the sensitivity of bacteria to other chemotherapeutic

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\* To prevent overgrowth of the whole plate by spreading *Clostridia*, the agar was divided by means of ditches into six segments and one strain seeded on each.

drugs, e.g. penicillin, propamidine and sulphone V. 187. The addition of lysed blood is not necessary with these drugs since they are not antagonised by substances in the medium.

### Summary

Most nutrient culture media contain sulphonamide-antagonising substances which complicate the in-vitro determination of sulphonamide sensitivity of bacteria. These substances can be neutralised by a factor present in the red blood cells of the horse, but not in human, rabbit or sheep cells. The nature both of the antagonisers and of the neutralising factor is unknown.

The addition of lysed horse blood to nutrient agar containing sulphonamide provides a solid medium which is stable and gives unequivocal evidence of sensitivity to known concentrations of sulphonamide.

We are greatly indebted to Dr Leonard Colebrook for allowing us to quote the results of his experiments with the capillary method. We also wish to express our sincere thanks both to him and to Dr R. E. O. Williams for their interest, advice and valuable criticism. We are indebted to the directors of the various laboratories that have sent samples of media for this investigation. The photographs are the work of the hospital photographic department.

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## NOTES ON THE EXAMINATION OF EXUDATES FOR PATHOGENIC ANAEROBES

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THE isolation and differentiation of anaerobes present two difficulties: in the first place cultivation on the surface of solid media requires complicated apparatus and is "tricky" and time-consuming, and secondly the recognition of organisms by their colony characters in deep cultures in solid media is not easy. The reason for the first of these difficulties is self-evident but the second depends on factors which are not appreciated by many workers. Of these the following demand special note. (i) If the conditions of so-called anaerobiosis—actually potency of electrons in the medium, referred to as Eh—are inadequate, typical colonies do not develop. (ii) The stiffness of the agar, as well as its nutrient properties, is not without influence on colony characters. For these reasons great variation in colony type may occur and colonies whose form is common to many anaerobes—"colonies en grenade" or "colonies en cœur jaune" of Weinberg—make recognition of differential characters impossible.

The following notes describe procedures which overcome these difficulties to some extent. They may be of special value to those who have had but little experience of anaerobes and who are so placed that the use of special apparatus is inconvenient. All procedures are carried out in the presence of air and the employment of tubes sealed with vaseline or paraffin is reduced to a minimum—a matter of importance to the laboratory technician. No claim is made to originality, for the methods used have already been described by others, notably Cruickshank and MacDonald (1942), and it is only their specific application to the study of pathogenic anaerobes that is stressed.

### I. *Culture in agar plates*

While any good nutrient agar can be used, it is wise for a worker when he has accustomed himself to a given medium to adhere to it. In the experience of the writer the more satisfactory media are those prepared from meat extracts, as colony development is better, although Fildes's CCY formula can quite well be used. The setting quality of the medium is important and should be standardised, at

least approximately. For this, Jenkins's (1921) method is employed and the jelly should be slightly less stiff than for surface cultures. A jelly of consistency corresponding to one instead of two tubes above the "flopping point" on Jenkins's scale is optimum.

Petri plates of 4 cm. diameter and 15 mm. depth are convenient and economical, as, when 15 c.c. of agar are poured into them, a depth of about 12 mm. is obtained. If such plates are not available truncated sputum jars with cotton wool plugs can be made to serve the purpose, the quantity of agar being reduced according to the diameter of the jars.

## II. *Reagents used to give the required negative potential to media*

Many reagents can be used for this, but ordinary ascorbic acid tablets, as advised by Cruickshank and MacDonald, are useful because they are readily available and as sold are usually sterile. It is only necessary, if anaerobic media are needed hurriedly, to crush a tablet in a sterile tube with a sterile glass rod and transfer a knife point of the powder to 10-15 c.c. of medium, but naturally it is better to prepare solutions of pure ascorbic acid of suitable strength, sterilise by Seitz filtration and store in convenient quantities. Solutions of 5 per cent. of the tablets can be added in the volume of 0.5 c.c. to 10 c.c. of medium. In the case of agar the jelly is melted, cooled to 45° C. and the requisite quantity of the solution added.

If great accuracy is required (it is not really necessary) the strength of this or other reductant to be used in media can be determined thus. Five c.c. quantities of nutrient agar in  $6 \times \frac{1}{2}$  in. tubes containing enough methylene blue to give a frank blue tint are melted and cooled to 45-50° C. To these, varying quantities of the reductant are added and the tubes set in cold water, after which they are incubated at 37° C. After three hours' incubation there should be a sharply demarcated line 4 mm. from the surface, the medium being blue above and colourless below: there should be no further change for 18-24 hours, but by the third day the blue layer may have extended to 6 mm. from the surface.

Using ascorbic acid tablet solution as reductant the required range will be found to lie between 0.375 and 0.125 c.c. of a 5 per cent. solution added to 5 c.c. of medium and for thioglycollate between 0.076 and 0.125 c.c. of a 2.5 per cent. solution. The quantity of reductant which gives the effect described is chosen for routine work, but with neither of these reductants does a fair excess usually do any harm. It must be noted, however, that occasional strains of anaerobes do not tolerate thioglycollate.

## III. *Medium for fermentation tests*

Hiss's serum peptone water (5 c.c. in  $6 \times \frac{1}{2}$  in. tubes) tinted with brom cresol purple and containing 1 per cent. of the fermentable

substance is used. The reductant should be thioglycollate, as both *Cl. septicum* and *Cl. multifementans* produce acid in presence of solutions of ascorbic acid tablets and in absence of other fermentable substrate.

#### IV. Medium for iron milk and lead acetate tests

No reductant is required for the iron milk medium but it must be noted that thioglycollate, being a sulphur-containing compound, tends to discolour media containing salts of iron or lead. For these ascorbic acid is used.

#### V. Comparison of upper limit of growth of various anaerobes with lower limit of blue colour in uninoculated tubes containing methylene blue when ascorbic acid is used as reductant

An uninoculated tube of agar containing sufficient methylene blue to give a definite tint to the medium was incubated along with similar tubes that had been seeded with different anaerobes, all the tubes containing the same quantity of ascorbic acid. This allows of comparison between the lower limit of blue tint in the control and the upper limit of growth in the cultures. The results after 24 hours' incubation at 37° C. were as follows.

(i) Depth of blue tint . . . . .	4 mm. from surface
(ii) <i>Cl. multifementans</i> upper limit of growth . . . . .	2 " " "
(iii) <i>Cl. welchii</i> . . . . .	3 " " "
(iv) <i>Cl. sporogenes</i> . . . . .	4 " " "
(v) <i>Cl. septicum</i> . . . . .	4 " " "
(vi) <i>Cl. oedematiens</i> . . . . .	4 " " "

The growth of the organisms will itself induce a negative potential and, if the tubes are very heavily seeded, the upper limit of growth may be nearer the surface than if the inoculum is small. This is important, for unless the reductant maintains an adequate negative potential in the medium for a sufficient length of time, then, with a light inoculum, growth may not be obtained except in an inconveniently deep layer of agar. The maintenance of negative potential can be assessed by mixing nutrient agar and methylene blue in suitable concentration in presence of ascorbic acid, allowing it to set at once and incubating at 37° C. The results are shown in the accompanying table. From this it is seen that to maintain for five days conditions suitable for the growth of exigent anaerobes a depth of over 9 mm. of medium is required when ascorbic acid is the reductant. This should be long enough to allow of the development of those anaerobes which are important in pathology. The findings indicate that the minimum depth of agar permissible in plates to be used for isolation is 12 mm.



TABLE

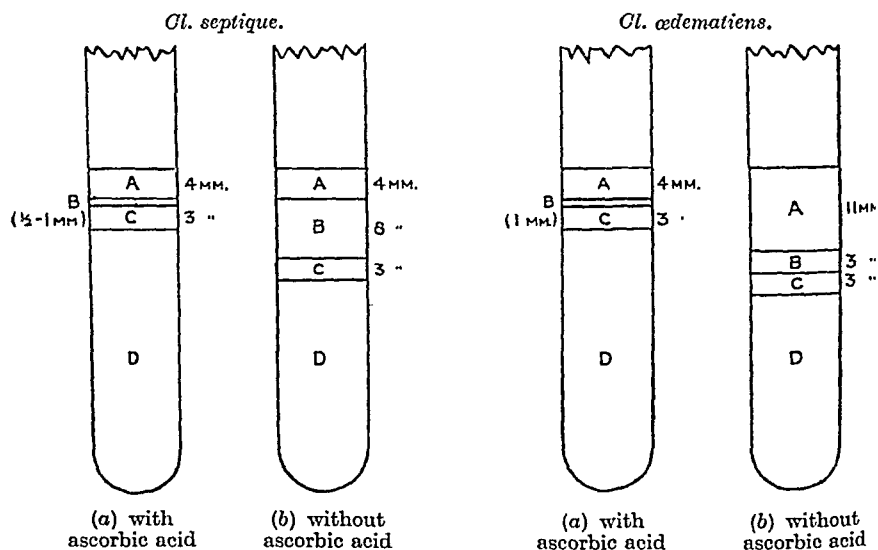
*Effect of ascorbic acid on the reduction of methylene blue in agar during incubation*

Time of incubation	Depth of blue colour from surface	
	With ascorbic acid	Without ascorbic acid
3 hours	4 mm.	11 mm.*
24 "	4 "	20 "
2 days	6 "	20 "
3 "	9 "	27 "
5 "	9 "	27 "
6 "	9 "	29 "

\* Below the blue layer the rest of the medium is light blue.

# VI. Comparison of growth in shake cultures of anaerobes with and without ascorbic acid in the medium

Tubes of nutrient agar were inoculated with *Cl. septicum* and *Cl. œdematiens* in duplicate, (a) with and (b) without the addition of ascorbic acid. The tubes were melted, cooled to 45° C., inoculated allowed to set and then incubated. The findings are shown in the accompanying figure. It is in zone B that "grenade" and "cœu



Growth of *Cl. septicum* and *Cl. œdematiens* in (a) ascorbic acid agar and (b) plain agar at varying depths of shake cultures. A = no growth, B = small colonies, C = very large colonies, D = "normal" colonies.

jaune" colonies, common to many anaerobes, are encountered and this zone is much restricted in tubes containing ascorbic acid agar

in place of plain agar. From this it will be appreciated that, in "fishing" colonies in deep agar plates, those near the surface should be avoided, and that ascorbic agar plates used to isolate anaerobes should be at least 12 mm. deep.

### VII. Routine procedure for examining wound exudates

1. Examine exudate microscopically.

2. Plate unheated exudate in ascorbic acid agar, using as average inoculum one  $\frac{1}{16}$  in. loopful in 15 c.c. of nutrient agar containing ascorbic acid cooled to 45° C. Of this, transfer one  $\frac{1}{16}$  in. loopful to a second 15 c.c. of melted ascorbic acid agar and pour both as plates in 4 cm. Petri dishes. Incubate as soon as the jelly has set. It is advantageous to add human serum to the agar—10 per cent. by volume—for if *Cl. welchii* be present, even in minimal numbers, the Nagler reaction allows of its detection with ease. The plating of unheated exudate should never be omitted, as the number of *Cl. welchii* in the sporulating state may be small.

3. Repeat (2) above with exudate heated to 65° C. for 45 minutes or 80° C. for 15 minutes.

4. Using the heated material, inoculate (i) a tube of milk containing ascorbic acid and (ii) a tube of Hiss's serum peptone water containing 1 per cent. salicin and either ascorbic acid or sodium thioglycollate. Tube (i) enhances the chance of isolating *Cl. welchii*, tube (ii) of *Cl. septique*.

5. Meat broth under vaseline is inoculated with the heated material at the same time as procedures 3 and 4 are carried out, so that the whole anaerobic flora may be preserved for future investigation if required.

It should be noted that, up to the present, no simple method has been devised for enriching the content of exudates so far as *Cl. tetani* and *Cl. oedematiens* are concerned.

### VIII. Colony "fishing"

To isolate, a characteristic colony, preferably deep in the medium, is selected, marked and "fished" with a sterile capillary pipette. It is then transferred to ascorbic acid broth, meat water under vaseline or ordinary broth containing a piece of iron, and incubated forthwith.

### IX. Differentiation

1. *Cl. welchii*. Colony discoid and Nagler reaction markedly positive. These features, with the morphology of the organism, usually serve to distinguish.

2. *Cl. septique*. Colony fluffy without black centre. "Fish" and inoculate into meat water, sucrose serum peptone water and

salicin peptone water. Sucrose is negative, salicin positive, meat is red and there is copious gas production.

3. *Cl. oedematiens*. The general appearance of colonies is lumpy, with a spiked edge, and the margins are denser than the centre after 48 hours. Fermentation tests with glucose, lactose, sucrose and salicin should be made and meat broth inoculated. Only glucose is fermented and meat broth becomes greyish in colour, with production of much gas. Verification by animal experiment is advisable.

4. *Cl. tetani*. The smaller colonies are "spidery" and the larger resemble colonies of *Cl. septique*. The development of terminal spherical spores and failure to ferment any sugar are suggestive but for an unequivocal diagnosis animal experiment is necessary.

5. *Cl. sporogenes*. Colonies have a characteristic dark centre and a feathery edge. Inoculated into meat water medium it produces gas with blackening and digestion of the meat. The cultures have a foul smell; glucose serum peptone water tubes give a soft clot which is soon digested.

If agglutinating sera are available these can be used to perform slide tests with cultures in ascorbic acid broth. Such tests have the advantage of being both rapid and specific.

#### X. Fermentation tests

Heavy inocula—two drops of either meat broth or ascorbic acid broth—are put into tubes of the requisite media, which, like the plates used for isolation, are incubated aerobically.

The complete series of tests involves the use of media containing glucose, sucrose and salicin, together with milk containing a piece of iron and serum peptone water to which lead acetate, 1 c.c. of a one per cent. solution per 100 c.c. of medium, has been added.

Using this schema isolation and differentiation have proved easy in the case of *Cl. welchii*, *Cl. septique*, *Cl. bifermentans* (*Cl. sordelli*), *Cl. histolyticum* and *Cl. multif fermentans*. We have also isolated *Cl. tetani* from the exudate of a wound containing *Cl. welchii*, *Cl. sporogenes* and *Cl. bifermentans*, but this was less easy. So far we have had no opportunity of testing the value of the procedure for isolating *Cl. oedematiens* from morbid material. What has been especially notable is the ease with which *Cl. sporogenes* can be avoided.

#### XI. Application of Petrie and Steabben's method to the deep ascorbic acid agar plate procedure

The method of Petrie and Steabben (1943), in which, by adding eight units of the requisite antitoxins to specially prepared media zones of specific precipitation are produced around the corresponding colonies, can be used with the deep plate technique. There are, however, certain points worthy of note in this connection.

1. The presence of antitoxic sera, all of which contain a small quantity of antibacterial antibodies, causes the colonies to be more dense than in simple media.

2. It is only colonies nearer to the surface which give haloes; many of these are of the "grenade" and "cœur jaune" type.

3. In the case of *Cl. oedematiens* the development of the zone of precipitate may be very slow, even when a surface plate is used, and with the deep plate method is so uncertain as to be of no value.

For these reasons the Petrie-Steabben procedure is of much less value in the deep plate method than when surface culture is used.

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576.8.097.29:576.851.57 (*Cl. welchii*)

## THE IN-VITRO PRODUCTION OF $\alpha$ TOXIN, $\theta$ HÆMOLYSIN AND HYALURONIDASE BY STRAINS OF *CL. WELCHII* TYPE A, AND THE RELATIONSHIP OF IN-VITRO PROPERTIES TO VIRULENCE FOR GUINEA-PIGS

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(PLATE XIII)

THE protective properties of *Cl. welchii* type A antiserum have previously been investigated in experimental gas gangrene produced in guinea-pigs by *Cl. welchii* A (Evans, 1943 *a* and *b*). It was found that antiserum containing either  $\alpha$  antitoxin and no  $\theta$  antihæmolysin or  $\alpha$  antitoxin and no antihyaluronidase was highly effective in protecting guinea-pigs against infection with a number of different strains of *Cl. welchii* A, while antiserum containing a considerable quantity of either  $\theta$  antihæmolysin or antihyaluronidase and only a trace of  $\alpha$  antitoxin was able neither to influence the course of the infection nor to enhance the protective action of  $\alpha$  antitoxin.

In the course of these investigations it was observed that the strains of *Cl. welchii* A employed differed in their power to produce fatal infection of guinea-pigs and also in their production in nutrient broth of the three antigens,  $\alpha$  toxin,  $\theta$  hæmolysin and hyaluronidase. It was considered that further information regarding the relative importance of these antigens in infection and also of their respective antibodies in the control of infection might result from a detailed examination of a larger number of strains of *Cl. welchii* A, by observing the relationship of their in-vitro properties to their in-vivo activity. A collection of 30 strains was therefore made, the majority coming from human sources, and each strain was examined for its property to produce, *in vitro*,  $\alpha$  toxin,  $\theta$  hæmolysin and hyaluronidase and also for its power to cause infection in guinea-pigs. In addition, this collection of strains was used for studying the hæmolytic activity of *Cl. welchii* A on horse blood agar and the protective action of the British Standard Gas Gangrene Antitoxin (*perfringens*) in experimental gas gangrene infection.

### EXPERIMENTAL

*Production of  $\alpha$  toxin and  $\theta$  hæmolysin in vitro.* Each strain was grown under the same conditions, using a peptone-salt-beef extract medium containing 0.45 per cent. of glucose (Gale and van Heyningen, 1942). With each strain

one litre of medium was inoculated with 5 c.c. of an 18 hours' liver-broth culture and incubated in a water-bath at 37° C. Throughout the period of growth a constant pH of 7.5 was maintained by the addition of alkali and the use of a glass electrode and potentiometer as in the method used by Gale and van Heyningen. Preliminary experiments showed that under these conditions the maximum amount of  $\alpha$  toxin and  $\theta$  haemolysin was produced after approximately 5 hours and at this time samples were taken and clarified by high speed centrifugation. The concentration of  $\alpha$  toxin in the clear supernatant fluid was estimated, using the lecitho-vitellin test (Macfarlane, Oakley and Anderson, 1941), by determining the number of units of  $\alpha$  antitoxin required to neutralise the  $\alpha$  toxin in 1 c.c. of supernatant fluid. In like manner the concentration of  $\theta$  haemolysin was estimated by determining the number of provisional units of  $\theta$  antihæmolysin (Evans, 1943a) required to neutralise the  $\theta$  haemolysin in 1 c.c. of supernatant fluid. This estimation was made with a suspension of sheep red cells of approximately 1.5 per cent. concentration; both the suspension and the dilutions of  $\theta$  antihæmolysin were made in isotonic M/15 phosphate buffer solution having a pH of 6.5, so that the hæmolytic activity of the  $\alpha$  toxin was completely inhibited (van Heyningen, 1941).

*Production of hyaluronidase in vitro.* In examining the hyaluronidase-producing properties of the strains, the same medium with the addition of 0.25 per cent. potassium hyaluronate was used, for McClean (1941) has shown that the inclusion of potassium hyaluronate in the culture medium gives a large increase in the yield of hyaluronidase. Each strain was grown for 18 hours at 37° C., when the pH of the culture fluid was adjusted to 7.5 and a clear supernatant fluid obtained by high speed centrifugation. The hyaluronidase concentration of the supernatant fluid was estimated by the mucin clot-prevention test (McClean, 1943) by determining the number of provisional units of antihyaluronidase (Evans, 1943b) required to neutralise the hyaluronidase in 1 c.c. of supernatant fluid.

*Ability of the strains to produce infection in guinea-pigs.* The method employed to produce experimental gas gangrene infection in guinea-pigs was similar to that devised by Armstrong and Rae (1941) and used in previous investigations (Evans, 1943 a and b). Into the shaven thigh of the left hind leg of the animal 0.2 c.c. of a 15 per cent. aqueous solution of  $\text{CaCl}_2$  was injected intramuscularly and 3 hours later 0.2 c.c. of a suspension of washed *Cl. welchii* A was injected into the same site. With each strain the suspension of washed bacilli was prepared in the same way. The organisms were grown in liver broth for 3 hours, the culture centrifuged and the deposit, after being washed once in saline, suspended in saline to give a concentration by measured opacity of  $250 \times 10^6$  organisms per c.c. From this suspension ten-fold dilutions were made in saline and 0.2 c.c. of the chosen dilution injected. No attempt was made to compare the infecting properties of the various strains by determining the smallest number of organisms required to produce fatal infection. A dose was chosen—0.2 c.c. of a  $10^{-3}$  dilution of the original suspension of  $250 \times 10^6$  organisms per c.c.—containing approximately  $50 \times 10^3$  organisms and with each strain this dose was injected into 6 guinea-pigs which were kept under observation for 7 days. With a number of the strains it was found that this dose produced fatal infection with unfailing regularity and the strains which behaved in this way were classed as virulent. With some of these virulent strains, even much smaller doses, such as 0.2 c.c. of a  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  and sometimes  $10^{-7}$  dilution, produced fatal infection. The remaining strains, which did not prove fatal when a dose of  $50 \times 10^3$  organisms was injected, gave a variety of reactions varying from slight local swelling at the site of injection to a large gangrenous lesion. These strains were further tested by injecting guinea-pigs with larger doses containing approximately  $50 \times 10^4$ ,  $50 \times 10^5$  and  $50 \times 10^6$  organisms. With these higher doses more deaths occurred and the gangrenous lesions in

the survivors were more widespread and severe. Only one of the strains, however, proved to be regularly fatal when a dose of  $50 \times 10^8$  organisms was given, and it was classed as moderately virulent; the remaining strains produced with this dose only an occasional death or no deaths at all and were classed as avirulent.

## RESULTS

### *Relationship of in-vitro properties to in-vivo activity*





























The results of these experiments, together with a statement of the origin of the strains are given in table I. All 30 strains produced  $\alpha$  toxin and they have been arranged in the table according to their ability to produce this antigen. A large variation was observed in the  $\alpha$  toxin-producing properties of the strains; the highest concentration of  $\alpha$  toxin was given by strain S 107, 1 c.c. of culture fluid requiring 1.6 units of  $\alpha$  antitoxin for neutralisation, while 5 of the strains produced only a trace of  $\alpha$  toxin, 0.02 unit of  $\alpha$  antitoxin being able to neutralise the toxin in 1 c.c. A similar variation was observed in  $\theta$  hæmolysin production; strain S 107 again gave the highest concentration while 10 of the strains produced none at all. Only 7 of the 30 strains produced hyaluronidase. On the basis of their in-vitro properties the strains can be broadly classified into three main groups; (i) those producing all 3 antigens, (ii) those producing  $\alpha$  toxin and  $\theta$  hæmolysin but no hyaluronidase and (iii) those producing only  $\alpha$  toxin. No strain has yet been encountered which produces  $\alpha$  toxin and hyaluronidase but no  $\theta$  hæmolysin.

These 30 strains of *Cl. welchii* were not chosen at random. Some were selected because of their good  $\alpha$  toxin-producing properties, others because they did not produce  $\theta$  hæmolysin and some because they were unable to cause fatal infection in guinea-pigs. Thus the distribution of in-vitro properties is not necessarily the same as that of a collection chosen at random. The object of the investigation was not to find out the most frequently occurring characteristics of strains of *Cl. welchii* A, but to study the relationship of in-vitro properties to in-vivo activity.

The examination of the infecting properties of the strains showed that 18 were virulent, being able to produce fatal infection in guinea-pigs with unfailing regularity when a dose of  $50 \times 10^3$  organisms was injected. Strain A 19 proved to be moderately virulent, a dose of  $50 \times 10^6$  organisms being required to produce fatal infection, while 11 of the strains were classed as avirulent since none was able to produce fatal infection regularly, even when a dose of  $50 \times 10^6$  organisms was injected. Table I shows, with each strain, the proportion of guinea-pigs dying after being injected with a dose of  $50 \times 10^3$  organisms and with the avirulent strains the proportion dying after the injection of  $50 \times 10^6$  organisms. As already mentioned, some of the virulent strains were also tested at lower doses and the results obtained with one of these— $50 \times 10^2$  organisms—are given.



TABLE I  
*In-vitro and in-vivo properties of 30 strains of Cl. welchii A*

Strain	In-vitro production of			Proportion of guinea-pigs dying after infection of			Virulent (+) Avirulent (-) for guinea-pigs	Source of strain
	$\alpha$ toxin	$\theta$ haemolysin	Hyaluronidase	$50 \times 10^3$ bacilli	$50 \times 10^3$ bacilli	$50 \times 10^3$ bacilli		
S 107				...	6/6	4/6	+	Intestine of sheep
SR 12				...	6/6	3/3	+	Gas gangrene
A 119			0	...	6/6	3/3	+	" "
A 117			0	...	6/6	3/3	+	" "
A 118				...	6/6	6/6	+	" "
SR 9			0	...	6/6	6/6	+	" "
Rosher			0	...	6/6	2/2	+	Puerperal gas gangrene
G 5g				...	6/6	...	+	Soil
3893			0	...	6/6	...	+	Wound—not gas gangrene
S 1a		0.0	0	...	6/6	2/2	+	" " "
BB		0.0	0	...	6/6	3/3	+	Vaginal swab: septic abortion
BS 1				...	6/6	4/4	+	Gas gangrene
3895		0.0	0	3/6	0/6	...	-	Wound—not gas gangrene



From the results shown in table I, it is evident that the power of a strain of *Cl. welchii* A to produce fatal infection in guinea-pigs is to a large extent related to its in-vitro  $\alpha$  toxin-producing properties. Of the 11 strains which proved to be avirulent, 9 produced only a small concentration of  $\alpha$  toxin, less than 0.05 unit of  $\alpha$  antitoxin being able to neutralise the  $\alpha$  toxin in 1 c.c. of culture fluid, while of the 21 strains which required more than 0.05 unit of  $\alpha$  antitoxin for neutralisation 18 were virulent, one was moderately virulent and only two were avirulent. It is also evident that virulence was not dependent on either  $\theta$  hæmolysin- or hyaluronidase-producing capacity, since 3 strains—S 1a, BB and 7731—which did not produce either of these antigens were found to be virulent, while 8 others which did not produce hyaluronidase were also virulent. On the other hand, strains such as D 3a, 4226 and 529 were avirulent, yet produced even more  $\theta$  hæmolysin but less  $\alpha$  toxin than some of the virulent strains. Up to the present, however, all the hyaluronidase-producing strains have proved to be virulent and no such strain has yet been obtained with poor  $\alpha$  toxin-producing properties.

Two of the avirulent strains—3895 and A 102—and the moderately virulent strain A 19, produced *in vitro* a concentration of  $\alpha$  toxin equal to and in some cases greater than the concentration produced by virulent strains. It is not to be expected, however, that in-vivo activity should be determined entirely by in-vitro  $\alpha$  toxin production. Other properties such as "invasiveness", a property not easily assessed, may be associated with the power of a strain to produce fatal infection.

#### *Hæmolytic properties of Cl. welchii type A on blood agar*

All the strains examined in the initial stage of this investigation were found to produce  $\theta$  hæmolysin in the beef extract medium and when grown on horse blood agar gave colonies surrounded by a zone of complete hæmolysis (fig. 1). When strain P 5706 was received from Dr Mary Barber, it was found to produce no  $\theta$  hæmolysin and no hæmolytic zone was detectable around colonies growing on horse blood agar. This strain did, however, produce  $\alpha$  toxin, which suggested that the zone of hæmolysis frequently present around the colonies of *Cl. welchii* A grown on horse blood agar was due to  $\theta$  hæmolysin and not to  $\alpha$  toxin. This suggestion was supported by growing, on horse blood agar containing (a) no antiserum, (b)  $\theta$  anti-hæmolysin and (c)  $\alpha$  antitoxin, a number of strains producing both  $\alpha$  toxin and  $\theta$  hæmolysin. With the medium containing no antiserum and that containing  $\alpha$  antitoxin a zone of complete hæmolysis surrounded the colonies after 24 hours' growth, but no hæmolysis occurred around the colonies grown on the medium containing  $\theta$  anti-hæmolysin. Although  $\alpha$  toxin hæmolyses the red cells of various species such as sheep, rabbit and guinea-pig, even when the calcium concentration

in the system is low, under similar conditions the red cells of goat and horse are practically insusceptible, and only partially susceptible when the calcium concentration is increased (Oakley and Warrack, 1941). Thus when strain P 5706, producing  $\alpha$  toxin but no  $\theta$  hæmolysin, was grown on horse blood agar which contained only a trace of calcium, the absence of hæmolysis was on account of the inability of  $\alpha$  toxin to hæmolyse the red cells. When however this strain was grown on blood agar to which had been added 0.5 per cent. of  $\text{CaCl}_2$ , the colonies obtained were surrounded by a wide zone of partial hæmolysis (fig. 2), and when strains producing both antigens were grown on this medium two zones of hæmolysis were observed, one narrow zone of complete hæmolysis and a much wider zone of partial hæmolysis extending beyond the boundary of the smaller (fig. 3). The zone of partial hæmolysis was produced by  $\alpha$  toxin and did not appear when  $\alpha$  antitoxin was present in the blood agar medium.

From this study of the cultural characteristics of *Cl. welchii* A on horse blood agar it was evident that strains not producing  $\theta$  hæmolysin could be identified easily and 9 of the 10 strains of this type used in these investigations were obtained from Miss Nancy Hayward's collection by merely selecting those which she had found to be non-hæmolytic on horse blood agar (Hayward, 1943).

#### *Protective action of the British Standard Gas Gangrene Antitoxin (perfringens)*

As previously shown by the lecitho-vitellin test, all the 30 strains of *Cl. welchii* A examined produced  $\alpha$  toxin neutralisable *in vitro* by  $\alpha$  antitoxin. The supernatant fluids from the broth cultures were tested also for toxicity by the intravenous inoculation of mice. It was found with all strains except A 78b, A 38b, D 3a and A 13b that at least 0.4 c.c. of the supernatant fluid was lethal and that the lethal effect could be neutralised in every case by the British Standard Gas Gangrene Antitoxin (*perfringens*) (Hartley and Evans, 1943). No attempt was made to determine by the mouse test the average lethal dose or the test dose of toxic supernatant fluids; the experiments were made merely to determine whether the standard serum was able to neutralise the toxin produced in culture by each of the strains.

Further experiments were made to determine whether the Standard Antitoxin was able to control the pathogenic action of each of these strains in experimental infection. With each strain the experiment was made in the same manner. Two groups of guinea-pigs, each of 2 animals, were used. The animals in one group each received a dose of standard serum containing 25 units of  $\alpha$  antitoxin subcutaneously just above the thigh which was to be infected. Three hours later all the animals in both groups were infected by the method already described, using for the virulent strains a dose

containing  $50 \times 10^3$  organisms and for the avirulent and moderately virulent strains a dose containing  $50 \times 10^6$  organisms. With all the strains except 3893 and 5053 the guinea-pigs receiving the standard serum were completely protected against death and infection (table II).

TABLE II

*Protective action of the British Standard Gas Gangrene Antitoxin (perfringens) in experimental gas gangrene produced by 30 different strains of Cl. welchii A*

Strain	Infecting dose of <i>Cl. welchii</i>	Result with guinea-pigs receiving			
		no serum		25 units of the British Standard	
S 107	Approximately $50 \times 10^3$ organisms	D 2	D 4	S—	S—
SR 12		D 3	D 3	S—	S—
A 119		D 1	D 2	S—	S—
A 117		D 2	D 5	S—	S—
A 118		D 2	D 2	S—	S—
SR 9		D 1	D 2	S—	S—
Rosher		D 2	D 4	S—	S—
G 5g		D 1	D 3	S—	S—
3893		D 2	D 2	D 2	D 5
SLa		D 3	D 4	S—	S—
BB		D 4	D 5	S—	S—
BSI		D 1	D 2	S—	S—
26		D 2	D 4	S—	S—
274		D 3	D 4	S—	S—
Mills		D 3	D 4	S—	S—
7731		D 4	D 5	S—	S—
PL		D 2	D 4	S—	S—
5053		D 1	D 3	S+	S++
A 19		D 5	D 6	S—	S—
3895		D 6	S++	S—	S—
A 102	Approximately $50 \times 10^6$ organisms	S++	S++	S—	S—
529		D 3	S+++	S—	S—
P 5706		S+	S+	S—	S—
Corcoran		D 8	S++	S—	S—
D 5		S+	S+	S—	S—
4226		S++	S++	S—	S—
A 78b		S+	S+	S—	S—
A 38b		S+	S+	S—	S—
D 3a		S++	S+	S—	S—
A 13b		S+	S+	S—	S—

Key to tables II and III: D 2 = death in 2 days; S = survival; — = no reaction; + = small gangrenous lesion; ++ = large gangrenous lesion; +++ = extensive gangrene.

With strain 3893, 25 units of  $\alpha$  antitoxin had little or no protective action against fatal infection, while with strain 5053, although the guinea-pigs receiving the serum were protected against death, they were not protected against infection. Further experiments were made with these two strains, using larger doses of standard serum, and it was found that 100-200 units of  $\alpha$  antitoxin were required for complete protection against fatal infection by strain 3893 while 50-100 units were necessary with strain 5053 (table III). It is not known why these two strains should have behaved differently from

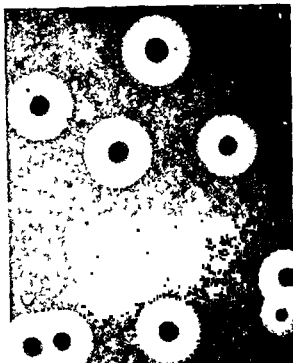
HÆMOLYTIC PROPERTIES OF *CL. WELCHII* TYPE A ON HORSE BLOOD AGAR

FIG. 1—Strain of *Cl. welchii* type A which produced  $\alpha$  toxin and  $\theta$  hæmolysin grown on horse blood agar, showing a well defined zone of complete hæmolysis  $\times 6$



FIG. 2—Strain of *Cl. welchii* type A which produced  $\alpha$  toxin but no  $\theta$  hæmolysin grown on horse blood agar to which had been added 0.5 per cent.  $\text{CaCl}_2$ , showing a wide zone of partial hæmolysis  $\times 6$ .

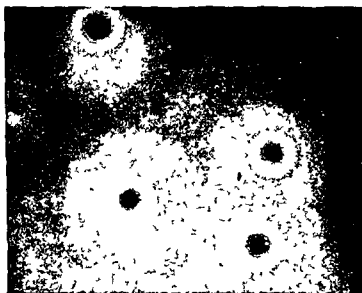


FIG. 3—Strain of *Cl. welchii* type A which produced both  $\alpha$  toxin and  $\theta$  hæmolysin grown on horse blood agar to which had been added 0.5 per cent.  $\text{CaCl}_2$ , showing a narrow zone of complete hæmolysis and a wider zone of partial hæmolysis  $\times 6$ .

containing  $50 \times 10^3$  organisms and for the avirulent and moderately virulent strains a dose containing  $50 \times 10^6$  organisms. With all the strains except 3893 and 5053 the guinea-pigs receiving the standard serum were completely protected against death and infection (table II).

TABLE II

*Protective action of the British Standard Gas Gangrene Antitoxin (perfringens) in experimental gas gangrene produced by 30 different strains of Cl. welchii A*

Strain	Infecting dose of <i>Cl. welchii</i>	Result with guinea-pigs receiving			
		no serum		25 units of the British Standard	
S 107	Approximately $50 \times 10^3$ organisms	D 2	D 4	S—	S—
SR 12		D 3	D 3	S—	S—
A 119		D 1	D 2	S—	S—
A 117		D 2	D 5	S—	S—
A 118		D 2	D 2	S—	S—
SR 9		D 1	D 2	S—	S—
Rosher		D 2	D 4	S—	S—
G 5g		D 1	D 3	S—	S—
3893		D 2	D 2	D 2	D 5
SIa		D 3	D 4	S—	S—
BB		D 4	D 5	S—	S—
BSI		D 1	D 2	S—	S—
26		D 2	D 4	S—	S—
274		D 3	D 4	S—	S—
Mills		D 3	D 4	S—	S—
7731		D 4	D 5	S—	S—
PL		D 2	D 4	S—	S—
5053		D 1	D 3	S+	S++
A 19	Approximately $50 \times 10^6$ organisms	D 5	D 6	S—	S—
3895		D 6	S++	S—	S—
A 102		S++	S++	S—	S—
529		D 3	S+++	S—	S—
P 5706		S+	S+	S—	S—
Corcoran		D 8	S++	S—	S—
D 5		S+	S+	S—	S—
4226		S++	S++	S—	S—
A 78b		S+	S+	S—	S—
A 38b		S+	S+	S—	S—
D 3a		S++	S+	S—	S—
A 13b		S+	S+	S—	S—

Key to tables II and III: D 2 = death in 2 days; S = survival; — = no reaction; + = small gangrenous lesion; ++ = large gangrenous lesion; +++ = extensive gangrene.

With strain 3893, 25 units of  $\alpha$  antitoxin had little or no protective action against fatal infection, while with strain 5053, although the guinea-pigs receiving the serum were protected against death, they were not protected against infection. Further experiments were made with these two strains, using larger doses of standard serum, and it was found that 100-200 units of  $\alpha$  antitoxin were required for complete protection against fatal infection by strain 3893 while 50-100 units were necessary with strain 5053 (table III). It is not known why these two strains should have behaved differently from

HEMOLYTIC PROPERTIES OF *Cl. welchii* TYPE A ON HORSE BLOOD AGAR

FIG. 1.—Strain of *Cl. welchii* type A which produced  $\alpha$  toxin and  $\theta$  haemolysin grown on horse blood agar, showing a well defined zone of complete haemolysis.  $\times 6$ .

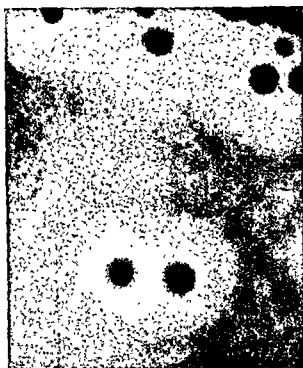


FIG. 2.—Strain of *Cl. welchii* type A which produced  $\alpha$  toxin but no  $\theta$  haemolysin grown on horse blood agar to which had been added 0.5 per cent.  $\text{CaCl}_2$ , showing a wide zone of partial hemolysis.  $\times 6$ .

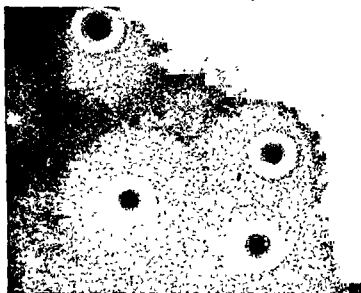


FIG. 3.—Strain of *Cl. welchii* type A which produced both  $\alpha$  toxin and  $\theta$  haemolysin grown on horse blood agar to which had been added 0.5 per cent.  $\text{CaCl}_2$ , showing a narrow zone of complete haemolysis and a wider zone of partial haemolysis.  $\times 6$ .





the others. From the in-vitro experiments it is seen that they did not give an exceptionally high concentration of  $\alpha$  toxin in culture,

TABLE III

*Protective action of the British Standard Gas Gangrene Antitoxin (perfringens) in experimental gas gangrene produced by an infecting dose of  $50 \times 10^3$  organisms of two strains of Cl. welchii A*

Dose of standard serum given to each guinea-pig	Result with guinea-pigs infected with strain			
	8893		5053	
200 units	S—	S—	S—	S—
100 "	S++	S+	S—	S—
50 "	D 4	S+++	S+	S+
25 "	D 3	D 4	S++	S+
Controls—No serum	D 2	D 4	D 2	D 2

but it is probable that they may have had a greater invasive activity and because of this a larger dose of antitoxin was necessary to cope with the infection they produced. In general, however, it can be stated that the British Standard Gas Gangrene Antitoxin (*perfringens*) was able to protect guinea-pigs against experimental infection produced by each of the 30 strains of *Cl. welchii* A examined, but that from 2 of the strains an infection developed which required a larger dose of  $\alpha$  antitoxin than was required in the case of the other strains in order to ensure complete protection.

#### SUMMARY AND CONCLUSIONS

Thirty strains of *Cl. welchii* A from a variety of sources have been examined for their ability to produce *in vitro*,  $\alpha$  toxin,  $\theta$  hæmolysin and hyaluronidase and for their power to produce experimental gas gangrene in guinea-pigs. Although all the strains were able to produce  $\alpha$  toxin, there was considerable variation in the amount produced by the different strains, a similar finding to that reported by other workers (Robertson and Keppie, 1941; Keppie and Robertson, 1944). The strains showed an even larger variation in their production of  $\theta$  hæmolysin and hyaluronidase, for a number of them did not produce these antigens at all. McClean and his collaborators (1943) have also reported that a number of strains examined by them were unable to produce hyaluronidase. The strains also differed considerably in their power to cause fatal infection in guinea-pigs.

The results of this investigation have shown that a general relationship exists between  $\alpha$  toxin production *in vitro* and ability to produce fatal infection in guinea-pigs, since strains producing only a small amount of  $\alpha$  toxin were unable to cause fatal infection, while the majority of those having better  $\alpha$  toxin-producing powers readily

produced fatal infection. On the other hand, the virulence of a strain did not depend on its ability to produce either  $\theta$  hæmolysin or hyaluronidase, for it was found that three strains which did not produce either of these antigens and eight others which did not produce hyaluronidase were no less effective in causing fatal infection in guinea-pigs than strains which produced both these antigens. These results, together with those obtained previously in the investigations on the protective action of  $\alpha$  antitoxin,  $\theta$  antihæmolysin and antihyaluronidase (Evans, 1943 *a* and *b*), suggest that  $\alpha$  toxin plays the most important part in gas gangrene infection with *Cl. welchii* A, and that  $\alpha$  antitoxin is the significant antibody in the control of the disease.

An examination of the hæmolytic action of *Cl. welchii* A on horse blood agar showed that the zone of complete hæmolysis surrounding the colonies of strains producing both  $\alpha$  toxin and  $\theta$  hæmolysin was a result of the activity of  $\theta$  hæmolysin and not of  $\alpha$  toxin (fig. 1), since no hæmolysis occurred when  $\theta$  antihæmolysin was present in the medium or with strains producing  $\alpha$  toxin but no  $\theta$  hæmolysin. When calcium was incorporated in the medium the horse red cells became partially susceptible to the hæmolytic action of  $\alpha$  toxin, with the result that a wide zone of partial hæmolysis appeared around the colonies of strains producing only  $\alpha$  toxin (fig. 2), while two zones appeared with the strains producing both  $\alpha$  toxin and  $\theta$  hæmolysin, namely a narrow zone of complete hæmolysis due to  $\theta$  hæmolysin and a wider zone of partial hæmolysis due to  $\alpha$  toxin (fig. 3). The hæmolytic properties of *Cl. welchii* A on horse blood agar made it possible to obtain a number of non- $\theta$  hæmolysin-producing strains for the investigation reported in the first part of this communication.

It has also been shown that the British Standard Gas Gangrene Antitoxin (*perfringens*) was able to protect guinea-pigs both against fatal infection with virulent strains and against infection with avirulent strains. Two of the virulent strains appeared to differ from the others in that a larger dose of antitoxin was required to ensure complete protection.

I am greatly indebted to Miss Nancy J. Hayward for the majority of the strains of *Cl. welchii* used in this investigation and to Dr D. McClean, Miss Helen E. Ross, Dr Mary Barber and Dr St John-Brooks for the others. I should also like to thank Mr V. Welch for the photographs.

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## LIPIDS OF THE HUMAN KIDNEY CORTEX AND MEDULLA IN FATTY CHANGE

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(PLATES XIV AND XV)

THE present investigation was undertaken to determine whether fatty change in the human kidney is the result of fatty infiltration or of fat phanerosis. Textbooks of pathology refer to fat phanerosis as being the sole mechanism of fatty change in the kidney. This theory was developed as a result of the work of Rosenfeld (1903), who could not demonstrate chemically an increased fat content of kidneys showing fatty change. It was inferred from his results that in fatty change the physico-chemical properties of the lipids of cytoplasm had altered in a way which rendered them histologically stainable. Hence the term "unmasking" of fats, or fat phanerosis. It is now generally accepted that fatty change in the liver is always accompanied by an increase in the neutral fat content of the organ. Dible (1934) and Dible and Gerrard (1938) have shown, contrary to the earlier doctrine, that fatty change in the myocardium is also an infiltration. It seemed reasonable to doubt the validity of Rosenfeld's results regarding fatty change in the kidney, especially since the results of Imrie (1914-15) also indicate that kidneys showing fatty change contain chemically more fat than the normal organ. Dible and Hay (1940) and Dible and Popják (1941) have investigated the fatty change produced in the rabbit's kidney by starvation. They found that in most cases this is confined to the loops of Henle and is an infiltration.

Because conclusive evidence from human material had not yet been obtained it was decided to investigate the question further, both morphologically and chemically.

### MORPHOLOGICAL INVESTIGATION

#### *Material and methods*

Kidneys from 221 non-selected post-mortems were fixed in formol-saline and cut on the freezing microtome at 15  $\mu$ , stained with Scharlach R and hæmatoxylin and mounted in glycerol-gelatin. Sections similarly cut and

mounted, but unstained, were also taken for examination with polarised light. These sections were examined within a few hours of mounting, since Duguid and Mills (1928) have shown that anisotropic granules will develop in frozen sections mounted in glycerol-gelatin after standing for a day or longer.

The degree of fatty change (microscopic fat content) was termed slight ( $\pm$ ) if only a few tubules in the particular part of the kidney were affected and the fat was in the form of fine granules; moderate (+, ++) if more tubules were affected, or the fat-droplets were larger, and marked (+++, +++) if most or all of the tubules contained large amounts of stainable fat.

### Results

The distribution of fatty change between cortex and medulla is shown in table I. It is evident that fatty change, especially when

TABLE I

*Distribution of fatty change between kidney cortex and medulla in 221 post-mortems*

Degree of fatty change	Cortex		Medulla	
	No. of cases	Percentage of total	No. of cases	Percentage of total
None . . . . .	151	68.3	131	59.7
Slight ( $\pm$ ) . . . . .	30	13.6	33	14.9
Moderate (+, ++) . . . . .	27	12.2	34	15.4
Marked (+++, +++) . . . . .	13	5.9	22	10.0
Total number of cases investigated .	221	100.0	221	100.0

marked, is more common in the medulla than in the cortex. In the former the loops of Henle, both the descending and ascending limbs, were affected. In cases of marked fatty change, the initial wide portion of the descending limb usually contained larger fat droplets (fig. 1), while the rest of Henle's loops contained only fine fat granules (fig. 2). This change was very similar to that produced in the rabbit's kidney by starvation (Dible and Hay, Dible and Popják). Moreover, in those cases in which fatty change occurred only in the medulla—the cortex being unaffected—starvation was a feature of the disease (carcinoma of the œsophagus, pyloric stenosis, diarrhoea and vomiting, and gastro-enteritis of infants) or merely a terminal phenomenon. Fatty change in the collecting and papillary tubules was found only once in this series, in the kidney of a child of 6 years who died of lymphatic leukaemia (fig. 3).

In order of frequency, after the loops of Henle the distal convoluted tubules in the cortex most commonly show fatty change. All the cases of slight and most of the cases of moderate (+) fatty change in the cortex were confined to the distal convoluted tubules (fig. 4). However, in cases of marked fatty change in the cortex, as is well known, the proximal convoluted tubules are affected (figs. 5 and 6);

## LIPIDS OF THE HUMAN KIDNEY



FIG 1—Fatty change in loops of Henle large fat droplets in cells of initial wide portion of descending loop Degree of fatty change in medulla ++++  $\times 550$  (Case P M 60 *cf* table VIII)

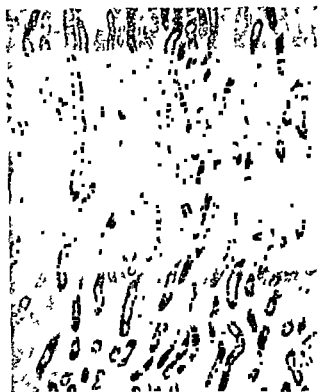


FIG 2—Fatty change in loops of Henle fine fat droplets in tubular epithelium Degree of fatty change in medulla +++  $\times 60$  (Case P M 1691, *cf* table IV)

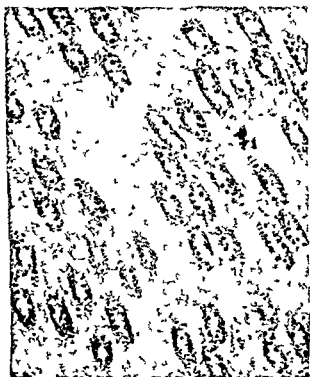


FIG 3—Fatty change in papillary ducts of kidney medulla  $\times 60$



FIG 4—Fatty change in distal convoluted tubules of kidney cortex Degree of fatty change in cortex  $\pm$   $\times 90$  (Case P M 1685, *cf* table III)





at the same time the distal convoluted tubules and loops of Henle are not necessarily involved. When, however, the tubules of the cortex contain large amounts of stainable fat, it is more common to find fat also in the loops of Henle, but it seems fair to say that the various portions of the tubules of the nephron can show fatty change independently of each other.

Fatty change in the cortex does not seem to be associated with starvation but with metabolic disorders like diabetes mellitus or toxic or septic conditions (toxæmia of pregnancy, phosphorus or aspirin poisoning, chronic abscesses, food poisoning, infectious diseases etc.) It seems likely, therefore, that in addition to the factor of fat mobilisation, as in starvation, another factor, which is not yet clear, is necessary for producing fatty change in the kidney cortex. Also it seems that the factors producing fatty liver and fatty kidneys are different. It is a common observation, and has been noted frequently during this investigation, that the liver might be loaded with fat and yet the kidneys be entirely free from stainable fat.

Anisotropic lipids were found in the epithelium of the convoluted tubules only in subacute and chronic nephritis and in essential hypertension. Chemical determinations carried out on a few such kidneys showed an increase in the ester cholesterol content of the organ. In cases of fatty change from other causes there was no anisotropic lipid material in the tubular epithelium of the cortex. In no case were there any anisotropic droplets in the epithelium of the medullary tubules. It seems, therefore, that in the kidney the same kind of fatty change can develop as in the myocardium or liver, thus might be termed the "simple fatty change of the kidney," i.e. uncomplicated by deposits of anisotropic lipids. For the systematic chemical investigations only this group of cases will be considered.

#### CHEMICAL INVESTIGATIONS,

To decide whether the fatty change in the kidney is the result of fatty infiltration or of fat phanerosis, chemical determinations of the fat content of the organ are necessary. If the change is an infiltration by mobilised depot fat, one should find an increased neutral fat content, if, however, it is a fat phanerosis, no such increase should occur.

#### *Material and methods*

For chemical fat determinations kidneys were obtained from post mortems performed not later than 24 hours and in most cases within 15 hours after death. All the bodies had been kept in the refrigerator.

As fatty change in the human kidney may occur independently in medulla and cortex, the fat content of these parts was determined separately in order to avoid dilution of the possibly increased fat content, for example, of a fatty medulla by the normal fat content of an unaffected cortex.

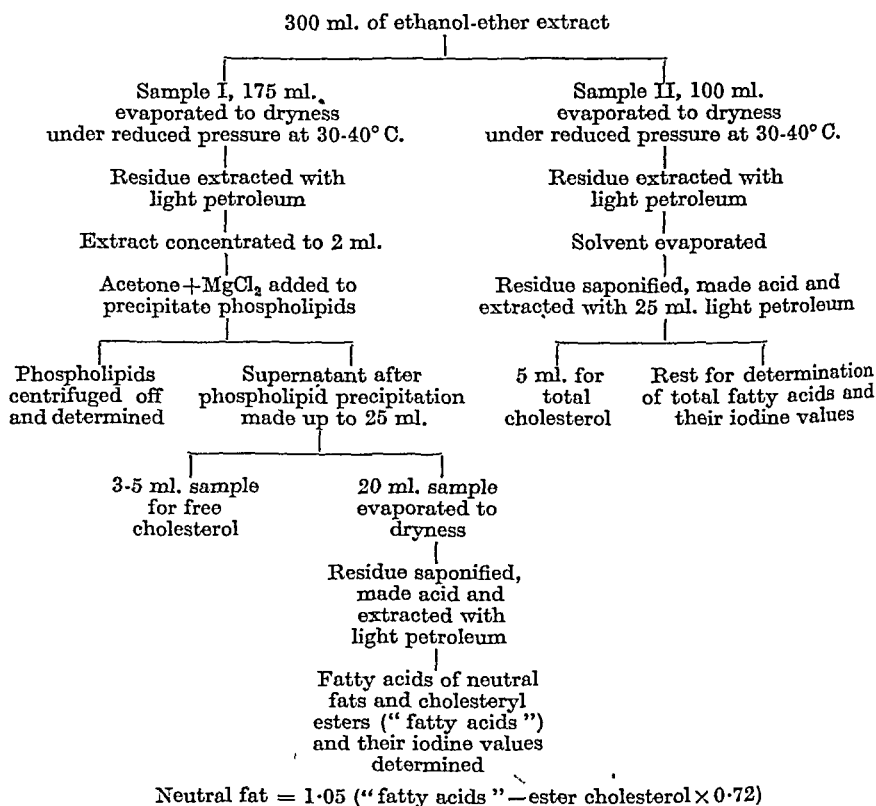
The renal capsule was stripped off and all pelvic fat removed. The organ was then cut up transversely into slices approximately 5 mm thick. The cortex was dissected with scissors from the medulla along the arcuate arteries, so

that most of the Henle's loops were included in the samples of medulla. For the determinations of the fat content of the medulla slices were selected in which the proportion of the zone of Henle's loops (the boundary zone) to the papillary portion of the medulla was the same. This selection seemed to be necessary because it was previously found in the rabbit's kidney (Dible and Popják) that the normal total fat content of the cortex determined by the method of Leathes and Raper (1925) was 1.8 per cent., of the boundary zone 1.4 per cent., and of the papillary portion of the medulla 0.8 per cent. of the moist weight. Since in the present investigation the boundary zone was not dissected from the rest of the medulla, in order to obtain comparable results in every case, it was important to choose slices of the medulla in which the proportion of the parts having a different fat content was approximately the same.

In the early part of the investigation the fats were determined by the saponification method of Leathes and Raper. The values so obtained represent total fatty acid plus total cholesterol content of the tissues, together with some unsaponifiable non-cholesterol lipid-soluble material. The amount of the latter in the kidney, however, is negligible. The iodine values were determined by the method of Dam (1924).

TABLE II

*Schematic representation of the steps involved in the isolation of the lipid fractions from an ethanol-ether extract of tissues*



In the later part of the investigation a detailed analysis of the lipid content of the kidneys was carried out. For these determinations micro-methods were

## LIPIDS OF THE HUMAN KIDNEY

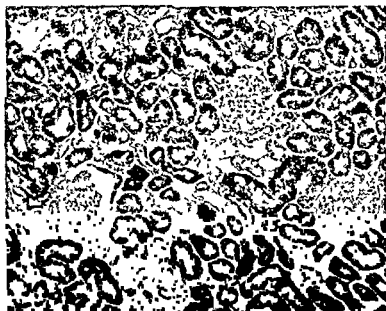


FIG 5—Marked fatty change (+++++) in kidney cortex from a case of phosphorus poisoning  $\times 110$ . (Case 20/42, cf table III)

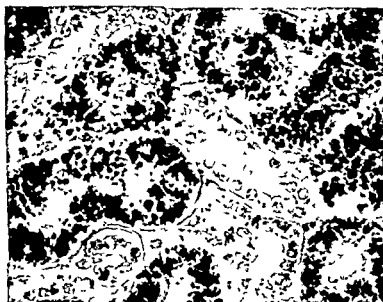


FIG 6—High power view of kidney cortex from the same case as fig. 5. It shows the proximal convoluted tubules full of fat granules, while the distal convoluted tubules are not affected.  $\times 550$ .



used and approximately 3 g. of fresh kidney cortex or medulla were sufficient. The tissue samples were extracted with boiling ethanol-ether (3 : 1 by volume) according to the method of Bloor (1929). For each g. of tissue at least 100 ml. of the solvent mixture were used. Table II gives the steps of the lipid analyses and the calculation of neutral fat content from values of fatty acids derived from neutral fats and cholesteryl esters ("fatty acids"). Phospholipids were determined by the method of Bloor (1929), fatty acids either by the dichromate oxidation method of Bloor (1928) or by titration with 0.02N NaOH. In the latter method it was assumed that the average molecular weight of the fatty acids was 275. The alkali titration method was used when the determinations had to be carried out on samples containing more than 5 mg. of fatty acids. The two methods yielded results differing by no more than 5 per cent. The cholesterol determinations were carried out by the digitonin method as described by Popják (1943). The iodine values in these micro analyses were determined by Yasuda's (1931-32) micro modification of Dam's method (table II).

### Results

The total fat content of the kidney cortex of adults, as determined by the method of Leathes and Raper, is shown in table III and that of the medulla in table IV. The normal fat content of the medulla is

TABLE III  
*Total fat content of kidney cortex of adults*  
(percentage of moist weight)

Case	Total fat content (per cent)	Iodine value	Microscopic fat content
Mean of 11 normal cases	$1.72 \pm 0.17$ (1.47)	$108 \pm 4$ (115)	0
P.M. 1687	1.72 (1.47)	108 (115)	±
" 1685	1.80 (1.65)	102 (108)	±
" 1701	1.89 (1.64)	103 (109)	±
" 1712	2.10 (1.85)	101 (106)	+
" 1691	2.65 (2.40)	96 (99)	++
" 1830	6.70 (6.45)	75 (75.3)	++++
" 1905	5.49 (5.24)	76 (76.5)	++++
" 12/42	6.35 (6.10)	75 (75.3)	++++
" 20/42	6.50 (6.25)	.	++++

In tables III and IV the determinations of fat content were made by the method of Leathes and Raper. Figures in brackets are corrected for total cholesterol and represent values of total fatty acids.

lower than that of the cortex by approximately 27 per cent. This is a finding similar to that of Dible and Popják for the rabbit's kidney.

A mild degree of histological fatty change ( $\pm$ ), either in the cortex or in the medulla, was associated with only a slight increase in the fat content. Indeed, the values in these cases are only the upper limits of the control figures. However, a moderate or marked fatty change is accompanied by an increase of approximately 50-300 per cent., according to the degree of fatty change observed histologically. It might be objected that the total fat content of the

cortex showing a ++++ fatty change was between 5.5 and 6.7 per cent., whereas that of the medulla showing the same degree of fatty change was only between 2.9 and 3.4 per cent. It should be

TABLE IV

*Total fat content of kidney medulla of adults  
(percentage of moist weight)*

Case	Total fat content (per cent.)	Iodine value	Microscopic fat content
Mean of 10 normal cases	$1.26 \pm 0.15$ (1.06)	$108 \pm 5$ (116)	0
P.M. 1698	1.42 (1.22)	...	±
" 1702	1.57 (1.37)	97 (101)	±
" 1685	1.46 (1.26)	92 (96)	+
" 1712	1.46 (1.26)	97 (101)	+
" 1701	1.72 (1.52)	92 (96)	+
" 1691	2.14 (1.94)	92 (95)	++++
" 20/42	2.28 (2.08)	...	++++
" 1830	2.93 (2.73)	...	+++++
" 12/42	3.16 (2.96)	85 (86)	+++++
" 1905	3.40 (3.20)	78 (79)	+++++

remembered, however, that only the loops of Henle in the boundary zone of the medulla showed fatty change and that the determinations were carried out on the whole medulla; therefore the increased fat content of the fatty part must have been diluted to a considerable extent.

From the results obtained by the method of Leathes and Raper it is not possible to tell which of the lipid fractions have increased in fatty change. The detailed lipid analyses were carried out to answer this question. The relevant results are presented in tables V to VIII. The data obtained from infants and young children up to the age of 2 years are presented separately from those obtained from adults, since infantile kidneys, both cortex and medulla, normally have a higher total fatty acid content than the kidneys of adults. This difference is due to the higher phospholipid content of infantile kidneys. The difference between the total fatty acid and "fatty acid" values in tables V to VIII represents the fatty acids of phospholipids. The theoretical fatty acid content of phospholipids is 69 per cent., and calculations based on the figures obtained in this investigation showed very good agreement with the theoretical value both in normal kidneys and in those with fatty change. The kidney cortex, as already pointed out, normally has a higher fat content than the medulla, both in adults and in infants, on account of its higher phospholipid content.

The results of these lipid analyses leave no doubt as to the nature of fatty change in the human kidney. With an increasing microscopic fat content, whether in the cortex or medulla of adults or infants,

there is an increase in the total fatty acid content of the tissues. The figures in the tables show that this increase is brought about by

TABLE V

*Lipid content of kidney cortex of adults  
(percentage of moist weight)*

Case	Total fatty acid		Fatty acid		Neutral fat	Microscopic fat content
	per cent	Iodine value	per cent	Iodine value		
Mean of 18 normal cases	1.55 ±0.17	116 ±8	0.51 ±0.15	136 ±13	0.51 ±0.14	0
P M 23	1.87	105	0.75	94	0.74	±
" 24	1.88	110	0.72	108	0.73	±
" 89	1.82	116	0.79	128	0.81	±
" 84	1.95	112	0.88	100	0.90	±
" 69	1.80	118	0.75	117	0.71	±
" 9	2.10	105	0.94	116	0.97	+
" 81	2.25	91	1.00	90	1.01	+
" 86	2.12		1.15	96	1.18	+
" 55	2.16		1.18	96	1.22	+
" 99	1.97	107	1.12	107	1.18	+
" 31	2.38	112	1.23	97	1.27	+
" 56	2.82	85	1.77	75	1.79	++
" 10	3.06		2.01	90	2.09	++
" W H	5.15	76	3.88	73	4.04	++++

TABLE VI

*Lipid content of kidney medulla of adults  
(percentage of moist weight)*

Case	Total fatty acid		Fatty acid		Neutral fat	Microscopic fat content
	per cent	Iodine value	per cent	Iodine value		
Mean of 24 normal cases	1.20 ±0.12	115 ±13	0.49 ±0.13	121 ±16	0.48 ±0.14	0
P M 46	1.35	105	0.64	108	0.65	±
" 31	1.65		0.62		0.64	±
" 9	1.57	104	0.69	100	0.71	±
" 84	1.64	103	0.84	108	0.84	±
" 24	1.65	120	0.74	118	0.76	±
" 89	1.51	115	0.76	112	0.78	±
" 78	1.60	117	0.85	105	0.88	±
" 23	1.93	94	1.07	93	1.10	+
" 81	2.26	99	1.31	78	1.34	++
" 56	1.88	90	1.30	78	1.33	++
" 99	2.79		1.67		1.73	+++
" W.H	2.87	87	1.75	89	1.87	+++
" 10	2.68	89	2.00	80	2.08	+++

an increase in neutral fat content. Neither the phospholipid nor the cholesterol content showed any significant deviation from normal in any of the cases.



The detailed analyses offered a definite advantage over those obtained by the method of Leathes and Raper. With the latter method, in cases of slight fatty changes a definite increase in fat content could not be satisfactorily demonstrated, as the individual

TABLE VII

*Lipid content of kidney cortex of infants and young children  
(percentage of moist weight)*

Case	Total fatty acids	"Fatty acids"	Neutral fat	Microscopic fat content
Mean of 7 normal cases	$1.87 \pm 0.30$	$0.42 \pm 0.09$	$0.42 \pm 0.09$	0
P.M. 99	2.09	0.81	0.83	+
" 52	2.41	0.94	0.95	+
" 45	2.03	0.85	0.89	+
" 47	2.26	0.97	0.99	+
" 60	2.56	1.20	1.24	++
" 21	2.66	1.40	1.45	++
" 117	3.65	2.15	2.20	+++

variations in the total fatty acids, influenced by the variations in total phospholipid and normal neutral fat content, obscured the slight increase. With the detailed lipid analyses, on the other hand, it was possible to demonstrate that even a slight fatty change will

TABLE VIII

*Lipid content of kidney medulla of infants and young children  
(percentage of moist weight)*

Case	Total fatty acids	"Fatty acids"	Neutral fat	Microscopic fat content
Mean of 3 normal cases	1.32	0.42	0.43	0
P.M. 94	1.90	0.77	0.79	±
" 67	2.11	0.75	0.76	±
" 45	1.52	0.65	0.66	±
" 107	2.29	0.97	1.01	+
" 93	1.94	0.80	0.80	+
" 117	2.84	1.66	1.71	++
" 21	2.47	1.64	1.71	++
" 47	2.72	1.69	1.75	++
" 99	2.79	1.67	1.75	++
" 52	3.70	2.69	2.79	+++
" 60	4.09	2.97	3.11	++++

produce about a 50 per cent. increase in the neutral fat content of the tissues, although such a definite increase is not evident from the total fatty acid values alone. Moderate degrees of histological fatty change (+, ++), in either cortex or medulla, resulted in a two- to

three-fold increase, and marked degrees of fatty change (+++, ++++) in a five- to eight-fold increase in the neutral fat content of the tissues. In tables III-VIII mean values for total fatty acid, "fatty acid" and neutral fat content of "fatty" kidneys are not given, since it is justifiable to calculate a mean value of these only for cases showing the same degree of fatty change. If a mean were obtained for the values of all cases, this would give a figure derived from a non-homogeneous group and would not express the true state of affairs.

An analysis of the iodine values of the total fatty acids and of "fatty acids" further substantiates the view that fatty change in the kidney is the result of infiltration of the parenchyma by neutral fats derived from the fat depots. It is well known that the iodine value of the fatty acids of body reserve fat, which is mostly neutral fat, is on the average only 60, while the iodine value of the fatty acids of the essential cell lipids is usually over 100. If fatty change is brought about by an infiltration with mobilised depot fat, one would expect a lowering of the iodine values of the neutral fat fatty acids in the affected organs and this should be proportional to the degree of infiltration. The lower iodine values of the neutral fat fatty acids should be also reflected in the iodine values of the total fatty acids.

In figs. 7-10, total fatty acid and "fatty acid" content of cortex and medulla are plotted against iodine values. The data of the curves have been calculated on the assumption that any increase in either total fatty acid or "fatty acid" content in fatty change is brought about by an infiltration with mobilised depot fat (iodine value of 60). The values of ordinates (iodine values) for the continuous curves have

been calculated by the equation  $Y = \frac{A \times B + 60X}{A + X}$  and for the dotted

lines by the equation  $Y = \frac{(A \pm \sigma)(B \pm \sigma^1) + 60X}{(A \pm \sigma) + X}$ , where  $A$  = mean normal total fatty acid, or mean normal "fatty acid" content;  $B$  = mean normal iodine value of total fatty acids or of "fatty acids";  $X$  = increase in total fatty acid or "fatty acid" content above normal values;  $\sigma$  = standard deviation of the normal total fatty acid or of "fatty acid" content;  $\sigma^1$  = standard deviation of the normal iodine values of total fatty acids or of "fatty acids"; and  $Y$  = theoretical iodine value. It can be seen from the figures that the observed iodine values agree with the theoretical within the expected limits of variation. The agreement of the observed iodine values with the theoretical is particularly good in the case of "fatty acids," as the slope of the initial part of the theoretical curves is very steep and thus even a slight degree of infiltration should result in a marked lowering of the iodine values; it is evident that this is the case. It should be mentioned, however, that such an analysis of the iodine values was possible only with the results obtained in adult kidneys. In infants the iodine values of both depot fat and the

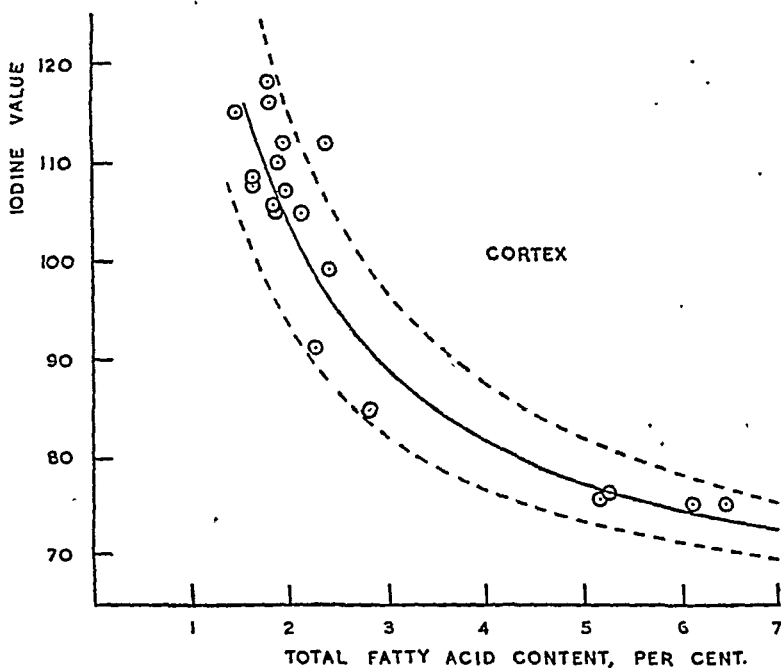


FIG. 7.

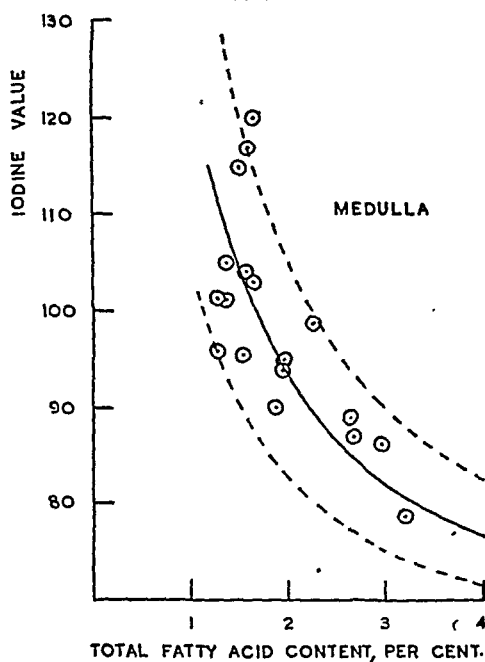


FIG. 8.

FIGS. 7-10.—Theoretical and observed iodine values plotted against total fatty acid and “fatty acid” content of kidney cortex and medulla. The theoretical curves have been obtained by assuming that the increased fat content was brought about by infiltration with mobilised depot fat having an iodine value of 60. The dotted curves were calculated by taking into account the limits of standard deviations, while in the calculations of the continuous curves the mean control values were used.

FIGS. 7 and 8.—Theoretical and observed iodine values of total fatty acids of adult kidney cortex and medulla respectively plotted against total fatty acid content.

fatty acids of the essential cell lipids varied with age between very wide limits and therefore it was impossible to obtain a mean for these values. This great variation in the iodine values in infants is presumably due to dietary differences and possibly to differences in the general fat metabolism as compared with that of adults.

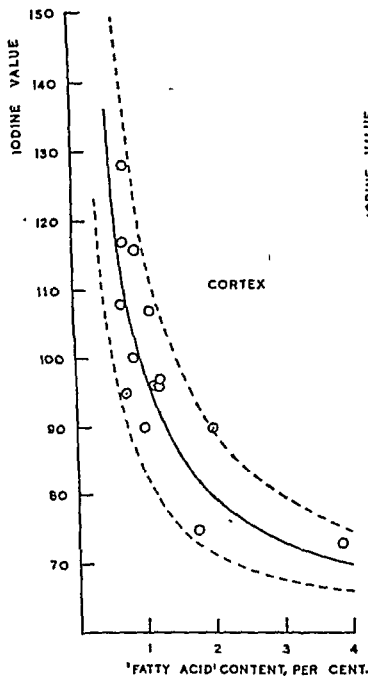


FIG. 9.

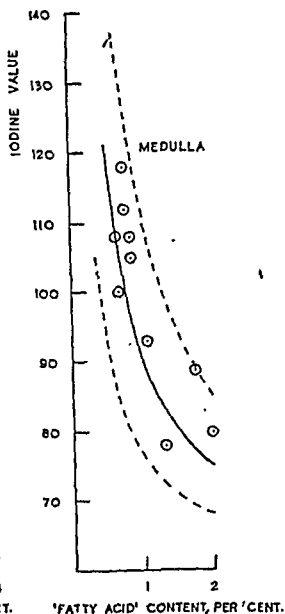


FIG. 10.

FIGS. 9 and 10.—Theoretical and observed iodine values of "fatty acids" of adult kidney cortex and medulla respectively plotted against "fatty acid" content.

The relationship between body reserve fat and fatty change provides further support for the view that fatty change is the result of fatty infiltration. Dible and his co-workers (*cf.* Dible, 1938-39) have shown that the degree of fatty change produced experimentally in the liver is a function of the body fat reserves; the same relationship was found in the case of fatty change produced in the rabbit's kidney by starvation (Dible and Popják). It is also well known from the work of Lebedeff (1883) and of Rosenfeld that fatty change in the human liver does not occur in phosphorus poisoning if there are no

fat reserves in the body. This correlation was noted in the present study too. In every case, except a few cases of slight ( $\pm$ ) fatty change, when fatty change occurred in the kidneys there were available fat reserves. To quote an example: cases P.M. 117 and 94 (cf. tables VII and VIII) were infants 5 and 3 months old respectively and both died of gastro-enteritis. In case P.M. 117, in which there was an abundance of depot fat, both liver and kidneys showed a moderate to marked degree of fatty change; whereas in case P.M. 94, in which the fat depots were depleted, apart from the slight fatty change ( $\pm$ ) in the medulla, neither liver nor kidneys contained more neutral fat than normal, although the cause of death and the duration of the preceding illness were the same.

## DISCUSSION

The data presented leave no doubt that fatty change in the kidney is of the nature of fatty infiltration, as this change resulted in an increased neutral fat content of the organ and the increase was proportional to the degree of fatty change observed microscopically. The changes in the iodine values corroborate this conclusion.

However, some might argue that even in cases of most marked fatty change in the kidney, the neutral fat content of the tissues did not exceed the value of about 5 per cent. of the moist weight, whereas in the liver a marked fatty change might yield as much as 15 per cent. of neutral fats. There is no difficulty in explaining this difference. When in the liver such large quantities of fat accumulate, usually each cell is occupied by a single large fat globule producing the characteristic signet-ring-shaped liver cells. The diameter of a large fat globule in a liver cell is on the average about  $40\ \mu$ . In the kidney, even in cases of the most marked fatty change, the diameter of the fat droplets seldom exceeds that of a red cell and is usually about  $3\text{--}5\ \mu$ . Only in the wide descending portion of Henle's loop were fat globules as large as  $10\ \mu$  in diameter observed. A fat globule  $40\ \mu$  in diameter, when broken up, could give rise to about 50 fat droplets of  $10\ \mu$  diameter and to 1000 fat droplets of  $4\ \mu$  diameter and consequently they would be dispersed through a larger volume than the original  $40\ \mu$  globule had occupied. The large number of small fat droplets in the tubular epithelium in the kidney gives the impression of a marked fatty change. Apart from this it is well known that even in cases of most marked fatty change in the tubular epithelium the fat granules still leave a good deal of cytoplasm unoccupied.

The chief difficulty which remains to be explained is the discrepancy between the results of Rosenfeld and those here presented. There are certain points in Rosenfeld's report which might account for his failure to demonstrate an increased fat content in "fatty" kidneys. Most of his work was carried out on dogs. His control

values were obtained on whole kidneys of animals which had been starved for 5 days. These showed total fat ranging from 18.5 to 29.12 per cent. of the dry tissues, which would correspond approximately to 4.6 per cent. of the moist weight. These values are much too high. It seems likely that many of Rosenfeld's animals developed fatty change in their kidneys during the 5 days of starvation. There is no evidence in his report that it was ascertained histologically that these kidneys were really normal. In the experimental production of "fatty" kidneys, certain poisons (phosphorus, phlorrhizin, chloroform, cantharidis, alcohol, pulegon) were given to the dogs and it seems to have been assumed *a priori* that these substances will produce the same degree of fatty change in the kidneys of all animals. No information is given in his article of the extent to which the kidneys of these experimental animals developed fatty change and whether it was the cortex or medulla which was affected. The fat content of the kidneys of his experimental animals varied from 16.9 to 22.6 per cent. of the dry weight, with a mean less than that of the controls.

It may be concluded, therefore, that, as in the liver and heart muscle, fatty change in the kidney is the result of infiltration of the parenchyma by mobilised depot fat. Thus all the available evidence shows that the theory of fat phanerosis is untenable and it should be replaced entirely by the concept of fatty infiltration. If it is agreed that fatty change in the parenchymatous organs is brought about by infiltration with fat derived from outside, there should no longer be any difficulty even in the use of the term "fatty degeneration", although not in its original sense as defined by Virchow. Degenerations result from injuries, which, although they do not kill the cell, alter its metabolism and morphological appearance. Degeneration may then be defined as the changes in the morphological appearance of the cell as a result of disturbed metabolism. Fatty degeneration in this sense of the term would be the morphological manifestation of an altered fat metabolism of the parenchyma cells.

#### SUMMARY

1. Fatty change was investigated in human kidneys both histologically and chemically.
2. An analysis of sections of kidneys from 221 post-mortems showed that fatty change occurs more frequently in the medulla than in the cortex. The various tubular portions of the nephron may show fatty change independently. Anisotropic lipids were found in the tubular epithelium of the kidney cortex only in subacute and chronic nephritis and essential hypertension.
3. Chemical analyses carried out separately on cortex and medulla showed that fatty change in the kidney is brought about by infiltration with neutral fat derived from the fat depots. Lipid fractions other

than neutral fats (phospholipids, free and ester cholesterol) showed no deviation from normal.

4. The kidney cortex has a higher phospholipid content than the medulla. Kidneys of infants, both cortex and medulla, contain more phospholipids than the corresponding parts of kidneys of adults.

5. The data presented show that the theory of fat phanerosis as an explanation of the mechanism of fatty degeneration is untenable and should be replaced by the concept of fatty infiltration.

I wish to express my gratitude to Professor J. H. Dible for initiating this work ; most of the kidneys forming the basis of the morphological investigations were obtained through his kindness. I am also indebted to Dr Eric Gardner for the case of phosphorus poisoning and for several sections of fatty kidneys.

The detailed data of this investigation have been deposited with the Librarian, General Library, British Museum (Natural History), London, S.W. 7.

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OBSERVATIONS ON THE ANTI-PERNICIOUS  
ANÆMIA FACTOR: WITH A SUGGESTED  
METHOD FOR TESTING LIVER EXTRACTS  
IN THE LABORATORYW. JACOBSON, Sir Halley Stewart Research Fellow, and]  
S. M. WILLIAMS \**From the Strangeways Research Laboratory, Cambridge*

(PLATE XVI)

THE chemical nature of the anti-pernicious anæmia principle is still obscure. The main reason for this is that there has been no reliable method of testing the hæmopoietic action of an extract other than clinically in a typical case of pernicious anæmia in relapse. So far pernicious anæmia has not been produced experimentally, and the bone marrow of normal animals will not respond to a hæmopoietic substance by forming more red blood cells. Numerous attempts have been made to devise a method of testing for the presence of the anti-pernicious anæmia principle. All failed to reveal a constant response to hæmopoietic extracts (Wintrobe, 1942, p. 276).

Two experimental methods of attack on this problem are described in the present communication: (1) the use of bone marrow cultures and (2) the use of splenectomised animals. The second and simpler method arose from observations on bone marrow cultures and has superseded the first method. The results of tests of clinically active substances by these techniques are reported.

## I. THE BONE MARROW CULTURE METHOD

Since normal bone marrow *in vivo* does not respond to the extra stimulus provided by the injection of hæmopoietic substances, it was decided to examine the reaction of bone marrow isolated in tissue culture.

## Technique

Bone marrow from adult rabbits and guinea-pigs was grown by either the hanging drop or roller tube method. Instead of the usual chick embryo extract a saline extract of the animal's minced liver was added to the plasma. This provided optimal conditions for erythropoiesis. A dilute saline extract of the animal's spleen was used in the control cultures; this medium did not stimulate erythropoiesis.

\* Working with a Lady Tata Memorial Trust grant.



## Results

The results can be arranged in two groups: (1) the response of bone marrow cultures to different fractions of liver extract and (2) the response to extracts prepared from different parts of the intestinal mucosa. These latter studies gave in addition information as to the anatomical localisation of the anti-pernicious anæmia substance.

### *Response of bone marrow cultures to liver extracts*

Fractionated liver extracts were supplied by Dr A. Neuberger. Remarkable differences could be established between some of these fractions when series of explants were grown in media containing them in progressive dilutions. Cultures grown with the addition of "active" extracts showed erythropoiesis even in dilutions of 1:300,000. Further fractionation of the active extracts was then undertaken by Dr Neuberger. In some cases the response was poor, but with the purest preparations it was possible to show the presence of a hæmopoietic factor in dilutions of 1:3,000,000. Some of these extracts were subsequently examined by the animal test and two fractions were also tried clinically. These observations gave consistent results. Unfortunately only a few milligrams of these fractions could be supplied at the time, and the method was discontinued as the simpler animal test was developed.

### *Localisation of the anti-pernicious anæmia substance in the intestinal mucosa*

Some doubt still exists as to the localisation of the anti-pernicious anæmia factor in the intestinal mucosa. Meulengracht (1934-35, 1939) suggested that the Brunner's glands of the duodenum elaborate this factor. Their structure is analogous to that of the pyloric glands. Another possibility is that the crypts of Lieberkühn are responsible, as they contain the argentaffine cells which are severely affected in cases of pernicious anæmia (Jacobson, 1939). In order to decide this question villi and crypts were separated from Brunner's glands by careful scraping of the pig's duodenal mucosa, the separation in layers being confirmed by histological examination.

Pieces of bone marrow which were grown with the addition of extract from the crypts showed erythropoiesis continuing as in cultures grown with the addition of liver extracts. Cultures grown with extracts of villi held an intermediate position, and those grown with extracts of Brunner's glands were almost or quite negative as regards erythropoiesis. These results indicate that Brunner's glands contain little if any hæmopoietic factor, and that the active principle is present in the crypts.

This interpretation agrees with the assumption that the argentaffine

cells in the crypts of Lieberkühn are connected with the elaboration of the active principle (Jacobson), since in Brunner's glands argentaffine cells are extremely rare. Further evidence for this assumption was provided by the study of bone marrow cultures grown *in vitro* with the addition of an extract of a carcinoid tumour from the human small intestine. A portion of the tumour measuring  $5 \times 4 \times 3$  mm. was minced and extracted with hot pyridine to dissolve out the pigment of the argentaffine cells. The pyridine was evaporated, and the dry matter (*circa* 0.5 mg.) re-suspended in 2 c.c. of saline. This extract intensely stimulated erythropoiesis in the bone marrow explants.

In a clinical experiment 64 g. of dried villi and crypts were given by mouth to a patient with pernicious anæmia, in 3 doses spread over a fortnight. After each dose, the percentage of reticulocytes in the blood rose on the 4th-6th day, and the red blood cell count increased from 3 to 3.9 million per c.mm. in 21 days. A subsequent injection of 2 c.c. of "Anahæmin" produced a reticulocyte peak of a similar order, though slightly smaller, as by then the patient was approaching a level of 4 million R.B.C. per c.mm.

In this connection, it is interesting that Wilkinson (1940) and his co-workers gave a large amount (699 g.) of Brunner's glands, isolated by the above method, to a patient with pernicious anæmia without producing any reticulocyte response, whereas a smaller amount (528 g.) of a fraction containing crypts and villi (which were not separated from each other) was found to be therapeutically active. This experiment supports the view that the crypts and villi, and not the Brunner's glands, contain the anti-pernicious anæmia factor.

## II. AN ANIMAL TEST FOR THE HEMOPOIETIC FACTOR

The following observations led to the use of splenectomised animals as a simpler test for the anti-pernicious anæmia principle. In the course of examining three cases of polycythæmia with red cell counts between 9 and 10 million per c.mm., sternal punctures were made in order to establish whether the disease in these patients was associated with mitotic over-activity of erythroblasts in the bone marrow. The marrow of all three was found to contain an excessive number of dividing erythroblasts.

Small fragments of this marrow were grown *in vitro* in media containing either liver or guinea-pig spleen extract. The liver extract enabled erythropoiesis to continue for three days *in vitro* (fig. 1), while the effect of the splenic extract was to inhibit erythropoiesis, so that from the second day on mainly young basophil erythroblasts were found (fig. 2). Intermediate stages such as polychromatic erythroblasts and normoblasts were almost absent. The splenic extract had no hæmolyzing effect on the red cells, with or without the addition of complement.

It therefore seemed likely that splenic extract acts as an inhibitor of erythropoiesis in the over-active bone marrow of the polycythæmic patient. Its effect on normal bone marrow cultures is similar, though less striking. This suggested two practical possibilities: (1) that the

giving of splenic extract to cases of polycythæmia might lead to a reduction in the number of red blood corpuscles (this has proved to be the case in all three patients in whom the treatment has so far been tried); (2) that removal of the spleen should enable normal bone marrow to respond to extra stimuli, *e.g.* the injection of hæmopoietic substances. (The bone marrow of a normal animal or man, or of a cured case of pernicious anæmia, will not respond to any amount of hæmopoietic substances.) The second assumption led to the use of splenectomised rabbits and guinea-pigs for testing the activity of hæmopoietic substances.

### Technique of the animal test

Twelve rabbits and two guinea-pigs were splenectomised and used as test animals. A thousand—frequently two thousand—red blood cells were counted for each animal daily, the number of reticulocytes being expressed as a percentage. Counts were made on both coverslips in an effort to avoid errors due to possible uneven distribution of the reticulocytes. These counts were done in groups of five hundred red blood cells under a  $\frac{1}{12}$  in. oil immersion objective and using a micrometer eyepiece.

First, daily reticulocyte counts were made on six normal rabbits before splenectomy, the periods of observation being respectively 5, 7, 25, 35, 49 and 93 days. During these periods the reticulocyte counts never rose spontaneously above 3.5 per cent. It was confirmed that normal rabbits cannot be used to test for the anti-pernicious anæmia factor. Four of these rabbits failed to respond to liver injections. The other two gave a positive followed by a negative response when a second injection was given after a prolonged interval; neither showed any anæmia.

### Results of the animal test

#### *General observations on splenectomised animals*

Full blood counts were done on all animals before splenectomy. It was found that the range of hæmoglobin and red cell counts in a series of seventeen rabbits before splenectomy was: Hb 79-110 per cent., R.B.C. 4,680,000-6,540,000 per c.mm. The corresponding values for a series of six guinea-pigs before splenectomy were: Hb 103-122 per cent., R.B.C. 5,200,000-6,460,000 per c.mm. Twelve of these rabbits and two of the guinea-pigs were used for the tests to be described.

Splenectomy does not appear to interfere in any way with the health of rabbits or guinea-pigs. In rats, as is well known, it is commonly followed by the development of anæmia associated with the presence of Bartonella in the red blood cells. Splenectomised rabbits and guinea-pigs do not become more susceptible to infection. Six of the splenectomised rabbits are still alive, and four of these were operated on two years ago. It should be emphasised that, though there may be a slight fall in Hb and R.B.C. after operation, due to loss of blood, neither a macrocytic nor a microcytic anæmia was ever found.

## CULTURES OF POLYCYTHAEMIC MARROW

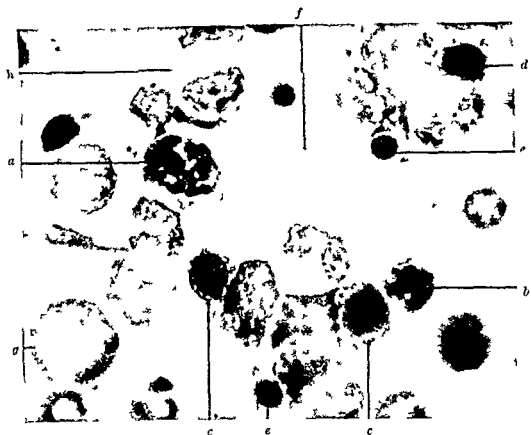


FIG 1—Smear from 3 day culture of polycythaemic bone marrow grown with liver extract Stain May Grunwald Giemsa (a) Pro erythroblast in early metaphase (b) Basophil erythroblast in early anaphase (c) Basophil erythroblast (d) Polychromatic erythroblast (e) Normoblast (f) Red blood corpuscle (g) Myeloblast (h) Myelocyte  $\times 600$



FIG 2—Smear from 3 day culture of polycythaemic bone marrow grown with the addition of spleen extract Stain May Grunwald Giemsa Group of five young basophil erythroblasts (pro erythroblasts) Intermediate stages of erythropoiesis were very rare  $\times 800$



The experiments described below are based on the stimulating action of the anti-pernicious anæmia factor on a bone marrow deprived of the inhibiting action of the spleen. No condition comparable with pernicious anæmia or any other deficiency disease of the bone marrow is produced by splenectomy in rabbits and guinea-pigs. After splenectomy in rabbits a reticulocyte "peak" of from 4 to 7 per cent. occurred about the 5th-9th day. The height of the peak varied with the amount of blood lost at operation, and consequently in some cases no peak was observed. After this, the reticulocyte counts gradually subsided to a normal level.

After repeated observations on the reticulocyte counts of these splenectomised animals, it was decided to take 3.5 per cent. as the upper limit of spontaneous variation. The normal reticulocyte values in splenectomised rabbits, after the post-operative period, varied between 1 and 3 per cent. Rarely values up to 3.5 per cent. were recorded, and this was taken as a safe "base line" for further experiments.

The two splenectomised guinea-pigs showed lower reticulocyte counts, most of them being below 1 per cent. However, they sometimes showed spontaneous rises to values between 2 and 3 per cent., lasting for about seven days. Similar though higher spontaneous rises, without apparent reason, have been recorded in the normal guinea-pig (Goodman, Geiger and Klumpp, 1936).

### Statistical analysis \*

#### *The reticulocyte percentage*

More than 3000 reticulocyte counts were made in the course of this work. The percentage of reticulocytes was calculated from counts of 1000 or 2000 red cells. Appreciable variation in the percentage of reticulocytes estimated in this fashion is to be expected on account of variations in distribution. The question arises whether the observed fluctuations are of the magnitude expected on the basis of the binomial distribution, and in particular whether reticulocyte percentages higher than 3.5 are found with appreciable frequency purely as a result of random sampling. If such were the case, some of the materials tested would be reported as active when in fact they were not active.

A rabbit was available for which 228 counts were made over a period of eight months. During this time eight injections were made. Seven of the substances used were inactive, while one was active. The reticulocyte percentages in the series of 228 determinations ranged from 0.8 to 3.9, and averaged 1.798. No spontaneous rise above 3.5 per cent. was observed. The value of 3.9 was recorded on the 5th day after the intramuscular injection of the only active

\* The authors are very grateful to Dr D. E. Lea for undertaking the statistical analysis.

substance given during this period of observation. The number of reticulocytes recorded per 1000 red cells are given in table I in the form of a frequency distribution.

TABLE I

*Frequency with which reticulocytes are found in a count of 1000 red cells*

Number of reticulocytes in 1000 red cells $r$	Expected frequency on binomial distribution $N \left( \frac{n!}{r!(n-r)!} \right) q^{n-r} \cdot p^r$	Frequency found experimentally
$\leq 7$	0.6	0
8	0.9	1
9	1.9	1
10	3.4	7
11	5.5	6
12	8.3	8
13	11.6	10
14	15.0	11
15	18.0	26
16	20.3	21
17	21.6	25
18	21.6	17
19	20.4	21
20	18.3	23
21	15.6	7
22	12.7	12
23	9.9	11
24	7.4	6
25	5.3	1
26	3.6	2
27	2.4	2
28	1.5	2
29	0.9	1
30	0.6	3
31	0.3	0
32	0.2	2
$\geq 33$	0.2	2*
Total number of counts made	228.0	228

\* Both these values were found on the 4th and 5th day after the intramuscular injection of an active liver extract.

If sampling errors alone accounted for the variation obtained, we should expect the number of counts in which  $r$  reticulocytes were found among  $n$  red cells to be given by the coefficient of  $p^r$  in the bi-nomial expansion of  $N(q+p)^n$ , where  $N = 228$ ,  $n = 1000$ ,  $p = 0.01798$ ,  $q = 0.98202$ . The calculated frequencies are included in the table, and are seen to be in good general agreement with the experimental frequencies. The agreement was tested by the  $\chi^2$  method, giving  $\chi^2 = 18.8$  for 15 degrees of freedom, indicating satisfactory agreement.\*

\* For the  $\chi^2$  test, values of  $r < 10$  were grouped as a single class, and values of  $r \geq 26$  as a single class, to avoid having classes of fewer than five members.

On the basis of the binomial distribution we should expect to obtain a reticulocyte percentage as great as or greater than 3.5 per cent. only once in about 5000 counts. In this rabbit one count of the 228 counts was higher than 3.5 per cent. (namely 3.9 per cent.) and was presumably due to the activity of one of the substances injected. We conclude that a peak in the reticulocyte percentage curve which rises above 3.5 per cent. is most unlikely to be due to chance.

### *The reticulocyte peak*

A substance is regarded as active only if the reticulocyte percentage rises above 3.5 per cent. on one (or more) of the following days, namely 2-6 days after an intravenous injection or 4-6 days after an intramuscular injection (table II). This convention might result

TABLE II

*Days after injection on which reticulocyte peaks occurred*

	Days after injection	Number of injections which have first peak at time stated	
Intravenous injections	1	0	Mean time at which peak appears = 4.0 days
	2	5	
	3	23	
	4	7	
	5	3	Most probable time at which peak appears = 3 days
	6	5	
	7	0	
	> 7	3	
Intramuscular injections	3	0	Mean time at which peak appears = 5.2 days
	4	2	
	5	5	
	6	4	Most probable time at which peak appears = 5 days
	> 7	0	

in some substances being reported as inactive which do give peaks, though not at the usual times. No liver extract produced a reticulocyte peak on any day other than those above-mentioned. In testing liver extracts this was found a very satisfactory criterion.

In view of the conclusion that a single count above 3.5 per cent. was only likely to occur about once in 5000 counts, spurious activity due simply to sampling error is not likely to occur more than about once in 1000 substances tested if the above convention is adopted. This value applies to a single test of one substance only, but when the same substance is tested twice and found active in both tests the chances of a sampling error are reduced still further.



### Experiments

Injections of hæmopoietic substances were given intravenously, intramuscularly, or (in a few cases) by stomach tube. When administered intravenously, a potent extract usually gave a reticulocyte peak between the third and fifth days, rarely on the second or sixth day; if a potent extract was given intramuscularly or orally, the reticulocyte response occurred between the fourth and tenth and usually between the fourth and sixth days. The reticulocyte values fell to a basal level within 6-10 days after intravenous injection, and within 8-14 days after intramuscular injection. The rabbits were then ready for a further test injection, but the guinea-pigs required a slightly longer interval between injections.

Repeated injections of hæmopoietic substances into the same animal at suitable intervals produced consistent results, and the six animals in their second year after splenectomy responded as satisfactorily as in the first few months.

Preliminary experiments were done with liver extracts of known potency in pernicious anæmia, in order to establish the fact that a reticulocyte peak could be produced consistently in the splenectomised animal. A maximal reticulocyte rise of 5-6 per cent. was obtained and could be reproduced after the injection of a human therapeutic dose of liver extract. It should be pointed out that a reticulocyte peak of 5-6 per cent. on a red cell count of 5-6 million is quite considerable.

Five liver extracts known to contain the anti-pernicious anæmia factor were tested.

**Liver extract I** (66 mg. dry matter per c.c.). Two c.c. gave, in a case of pernicious anæmia with R.B.C. of 1,020,000 and Hb 25 per cent., a reticulocyte response of 30.2 per cent. on the fourth day after injection. The same dose injected intravenously into three splenectomised rabbits produced reticulocyte peaks of 5.9, 5.8 and 5.5 per cent. (A. 1, fig. 3) respectively, and in a fourth rabbit 1.7 c.c. produced a peak of 5.1 per cent. (A. 2, fig. 4); 0.8 c.c. injected into three of these four rabbits produced peaks of 3.4, 3.8 (A. 3, fig. 3) and 3.9 per cent. (A. 3, fig. 4); 0.5 c.c. gave peaks of 4.0, 3.8 (A. 4, fig. 3), 4.3 and 3.1 per cent. (A. 4, fig. 4). From these values it was concluded that the smallest dose giving any significant rise lay between 0.5 and 0.8 c.c. Two c.c. gave a nearly maximal response. Larger doses given intravenously were found unsatisfactory. It was suspected that the amount of antiseptic present in the ampoules might inhibit the reticulocyte response, as each c.c. contained 3 mg. of tricresol. Thus 3.5 c.c. of this extract, containing 10.5 mg. tricresol, failed to produce a reticulocyte response, whereas the same amount of dry matter (235 mg.) without any disinfectant produced a reticulocyte peak. Two guinea-pigs showed reticulocyte peaks of 4.1 and 3.6 per cent. respectively after injections of 60 mg.

**Liver extract II.** Two hundred mg. of the dry extract were dissolved in 4 c.c. of saline and filtered through a sterilising Seitz filter pad (3.5 cm. diameter) to avoid possible infection. This extract failed to produce any reticulocyte response. That the active matter was adsorbed on to the filter pad was shown by preparing the extract in the same way, but injecting it

immediately without filtering. This gave a reticulocyte peak of 5.9 per cent. on the fifth day (fig. 5).

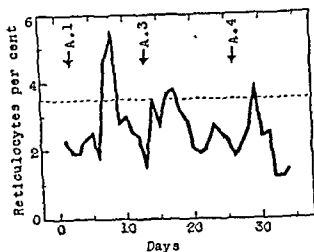


FIG. 3.—Reticulocyte response of splenectomised rabbit to the intravenous injection of extract I.

A. 1 = 2.0 c.c. of extract injected  
A. 3 = 0.8 " " " "  
A. 4 = 0.5 " " " "

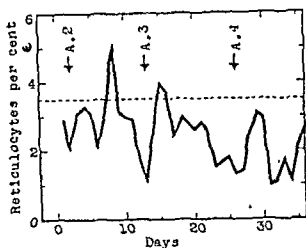


FIG. 4.—Reticulocyte response of splenectomised rabbit to the intravenous injection of extract I.

A. 2 = 1.7 c.c. of extract injected  
A. 3 = 0.8 " " " "  
A. 4 = 0.5 " " " "

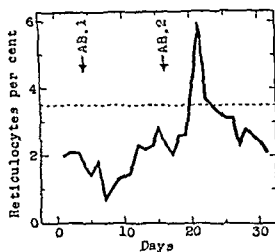


FIG. 5.—Reticulocyte response of splenectomised rabbit to the intravenous injection of extract II.

AB. 1 = 200 mg. of filtered extract injected.  
AB. 2 = 200 mg. of unfiltered extract injected.

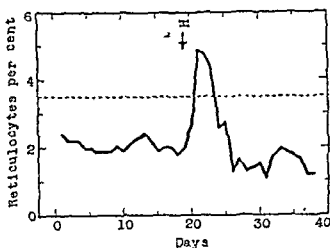


FIG. 6.—Reticulocyte response of splenectomised rabbit to the intravenous injection of extract III.

H = 125 mg. of extract injected.

**Liver extract III.** Two rabbits each injected with 2 c.c. [= 50 mg.] gave reticulocyte values of 4.1 and 3.9 per cent. respectively. But another rabbit injected with 3 c.c. of this extract failed to give a significant response. On the six days preceding the injection the average reticulocyte count in this animal was 1.3 per cent. [range 1.1-1.6]. On the fifth day after injection the reticulocyte count was 3.2 per cent., and on the two following days 3.0 and 3.1. On the 6th day after injection the R.B.C. count was 7,150,000, an unusually high value. One hundred and twenty-five mg. produced a peak of 4.8 per cent. (fig. 6). Two c.c. of this extract [= 50 mg.] produced a reticulocyte peak of 15.1 per cent. on the fifth day in a case of pernicious anaemia with a R.B.C. count of 1,500,000 per c.mm. It was thus evident that 50 mg. of extract III

### Experiments

Injections of hæmopoietic substances were given intravenously, intramuscularly, or (in a few cases) by stomach tube. When administered intravenously, a potent extract usually gave a reticulocyte peak between the third and fifth days, rarely on the second or sixth day; if a potent extract was given intramuscularly or orally, the reticulocyte response occurred between the fourth and tenth and usually between the fourth and sixth days. The reticulocyte values fell to a basal level within 6-10 days after intravenous injection, and within 8-14 days after intramuscular injection. The rabbits were then ready for a further test injection, but the guinea-pigs required a slightly longer interval between injections.

Repeated injections of hæmopoietic substances into the same animal at suitable intervals produced consistent results, and the six animals in their second year after splenectomy responded as satisfactorily as in the first few months.

Preliminary experiments were done with liver extracts of known potency in pernicious anæmia, in order to establish the fact that a reticulocyte peak could be produced consistently in the splenectomised animal. A maximal reticulocyte rise of 5-6 per cent. was obtained and could be reproduced after the injection of a human therapeutic dose of liver extract. It should be pointed out that a reticulocyte peak of 5-6 per cent. on a red cell count of 5-6 million is quite considerable.

Five liver extracts known to contain the anti-pernicious anæmia factor were tested.

**Liver extract I** (66 mg. dry matter per c.c.). Two c.c. gave, in a case of pernicious anæmia with R.B.C. of 1,020,000 and Hb 25 per cent., a reticulocyte response of 30.2 per cent. on the fourth day after injection. The same dose injected intravenously into three splenectomised rabbits produced reticulocyte peaks of 5.9, 5.8 and 5.5 per cent. (A. 1, fig. 3) respectively, and in a fourth rabbit 1.7 c.c. produced a peak of 5.1 per cent. (A. 2, fig. 4); 0.8 c.c. injected into three of these four rabbits produced peaks of 3.4, 3.8 (A. 3, fig. 3) and 3.9 per cent. (A. 3, fig. 4); 0.5 c.c. gave peaks of 4.0, 3.8 (A. 4, fig. 3), 4.3 and 3.1 per cent. (A. 4, fig. 4). From these values it was concluded that the smallest dose giving any significant rise lay between 0.5 and 0.8 c.c. Two c.c. gave a nearly maximal response. Larger doses given intravenously were found unsatisfactory. It was suspected that the amount of antiseptic present in the ampoules might inhibit the reticulocyte response, as each c.c. contained 3 mg. of tricresol. Thus 3.5 c.c. of this extract, containing 10.5 mg. tricresol, failed to produce a reticulocyte response, whereas the same amount of dry matter (235 mg.) without any disinfectant produced a reticulocyte peak. Two guinea-pigs showed reticulocyte peaks of 4.1 and 3.6 per cent. respectively after injections of 60 mg.

**Liver extract II.** Two hundred mg. of the dry extract were dissolved in 4 c.c. of saline and filtered through a sterilising Seitz filter pad (3.5 cm. diameter) to avoid possible infection. This extract failed to produce any reticulocyte response. That the active matter was adsorbed on to the filter pad was shown by preparing the extract in the same way, but injecting it

# ISO-AGGLUTININ TITRES IN HETEROSPECIFIC PREGNANCY

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THE discovery of the rhesus factor (Landsteiner and Wiener, 1940) and its association with hæmolytic disease of the newborn (erythroblastosis foetalis) (Wiener and Peters, 1939-40; Levine, Katzin and Burnham, 1941) have renewed interest in the older theories of Dienst (1905) and Hirszfeld (1928), that an incompatibility between the blood of mother and foetus might be responsible for toxæmia of pregnancy and much neo-natal mortality and morbidity. Thus, as early as 1905, the possibility that maternal iso-immunisation might be a factor in certain morbid conditions during pregnancy and the neo-natal period was recognised, but Dienst later retracted his views (1908) and the theory was discarded until Ottenberg (1923) reopened the question. Hirszfeld, reviewing the early work, used the terms heterospecific and homospecific pregnancy. By heterospecific pregnancy he meant a pregnancy where the child was shown to have a blood group different from that of the mother, while by homospecific pregnancy he meant one where the blood groups were the same. Later these terms were used in a narrower sense, a heterospecific pregnancy being defined as one in which the child exhibited a blood group antigen of the ABO system which was absent from the maternal red cells (Levine, 1943). This occurs in about one in five pregnancies, and in all such cases the mother's serum must contain an iso-agglutinin which can react with the child's cells *in vitro*, e.g. a group O mother with a group A foetus has both  $\alpha$  and  $\beta$  in her serum. We shall use the term in this restricted sense throughout.

With the discovery that Rh incompatibility is responsible for a very high proportion of cases of hæmolytic disease of the newborn, attention was directed towards the possibility that ABO group incompatibility might be the ætiological factor in cases in which Rh incompatibility could not be demonstrated. Observations by Boorman, Dodd and Mollison (1942) support the idea that anti-A or anti-B agglutinins may occasionally cause the destruction of foetal erythrocytes of group A or group B respectively, and thus be the chief ætiological factor in some cases of erythroblastosis foetalis.

It is supposed that, where incompatibility exists, iso-immunisation of the mother takes place by the abnormal passage either of foetal erythrocytes or of soluble group-specific substances present in the foetal tissue fluids through the placenta into the maternal circulation. Some explanation is of course necessary for the fact that the presence of natural anti-A or anti-B agglutinin in the serum of a mother carrying a baby of group A or B does not usually lead to any abnormality in the baby, whereas the presence of immune anti-Rh agglutinins is often associated with erythroblastosis in the Rh-positive infant. Wiener has suggested that it is because the Rh factor is found only in the erythrocytes, whereas the A and B group-specific substances are present in all the body tissues and secretions in the majority of individuals whose erythrocytes contain the A or B factor; they therefore neutralise any  $\alpha$  or  $\beta$  passing into the foetus and thus protect the erythrocytes from damage. Boorman and Dodd (1943) have, however, shown that the Rh factor is also present in the tissues and saliva, although in much smaller quantities than in the erythrocytes.

Since by heterospecific pregnancy we mean one in which the foetus carries a dominant blood-group gene absent from the maternal genetic constitution, the different combinations of blood groups which constitute a heterospecific pregnancy are as shown in table I.

TABLE I  
*Heterospecific pregnancies*

Blood group of mother	Blood group of child
O	A, B
A	AB, B
B	AB, A
AB	No heterospecifics

Theoretically, if iso-immunisation occurs in heterospecific pregnancy in a group O mother with a group A foetus, stimulation of maternal antibodies should occur, *i.e.* a group A foetus might be expected to exert an antigenic action in the mother and this might be capable of detection by an increase in the titre of anti-A ( $\alpha$ ) agglutinin in the maternal serum. It is known that such a phenomenon occurs in a group O person accidentally transfused with group A cells, and sometimes after the administration of group A serum or plasma (Aubert *et al.*, 1942). In order therefore to determine if this occurs in heterospecific pregnancy, we decided to test samples of serum at intervals during pregnancy, and to estimate the agglutinin titres of those samples throughout and following the pregnancy.

The material used was obtained from primiparae attending the ante-natal clinic of the maternity hospital, primiparae being chosen in order that the effect

of any previous pregnancy could not confuse the issue. The first sample was usually obtained about the end of the third or beginning of the fourth month of pregnancy, i.e. when the patient first attended. Samples obtained at fortnightly intervals and later at monthly intervals were considered sufficient; in some, only one sample was obtained at the first attendance and a further sample just after the confinement. The mother was asked to report back, where possible, in about six weeks, in order that a further sample could be obtained in the puerperium; in a few cases a sample of maternal serum was also obtained as long as a year after the confinement.

### *Procedure*

1. The maternal blood was grouped, and in all cases the presence or absence of the rhesus factor was noted.

2. The agglutinin titres of the maternal serum were estimated, using at all times the technique described below.

3. The patient's ante-natal card was stamped with the blood group and, when she was admitted in labour with such a stamped card, a sample of umbilical cord blood was collected in a heparinised bottle at the time of delivery and submitted to the laboratory for grouping.

A sample of blood from the mother was taken soon after the completion of labour. When obtaining samples of cord blood for grouping, care must be taken not to squeeze the cord lest the foetal blood become contaminated with Wharton's jelly and false agglutination results occur. It is also essential that the sample be not contaminated with blood from the surface of the placenta. In one instance a cord sample appeared to give the reactions of group AB cells; the mother had been proved to be group O and further investigation showed the child to belong to group B. The cord blood had been inadvertently contaminated with maternal blood in the labour room, with the result that the  $\beta$  agglutinin of the mother's serum had agglutinated the child's B cells, and therefore the cells were already agglutinated when the typing sera were added to the cell suspensions. If any doubt arises, the child's blood group should be verified from a sample obtained by puncturing the heel.

*Method of titrating the maternal serum.* Serial dilutions of serum were made as follows. An initial dilution of 1:10 was made by mixing 0.1 c.c. of serum with 0.9 c.c. of isotonic saline solution; of this 0.05 c.c. was transferred to a tube containing 0.05 c.c. of isotonic saline, giving therefore a dilution of 1:20 and so on by doubling dilutions until a final dilution of 1:320 was obtained. If this titre was exceeded, the titration was repeated up to 1:5120 or more if necessary. To each tube was added 0.05 c.c. of a washed suspension (2 per cent.) of pooled A or B cells (whichever agglutinin titre was being estimated). Pooled cells from 4-6 different people were used to obviate any variation in agglutinin content of the cells. Controls of these pooled cells in saline were put up every time new cells were used, together with standard serum controls. The tubes were allowed to stand at room temperature for 2 hours, after which readings were made and the agglutinin titres thus estimated. During the investigations this procedure was rigidly adhered to. The method and notation are very similar to those recommended in the Medical Research Council Report on blood grouping (1943).

### RESULTS

It was generally found that during heterospecific pregnancy there was a distinct rise in the maternal agglutinin titre corresponding to the child's blood group. At the same time, in group O mothers, the

titre of the other agglutinin showed a slight rise towards the end of term. These phenomena are comparable with the specific and non-specific rises in agglutinin titre which occur when a person who has been previously immunised against *Bact. typhosum*, *paratyphosum A* and *paratyphosum B* is infected with *Bact. typhosum*, the specific antibody showing a steep rise together with a slighter rise in the *paratyphosum A* and *B* agglutinins. In contrast, the cases of homospecific pregnancy examined, with one exception, showed no such rise of agglutinins. The one exception was a group O mother with a group O baby (case 7884), in which there was a rise in the  $\alpha$  titre of the maternal serum along with a less steep rise in the  $\beta$  titre. No explanation for this is available at present, except that the child may have been a weakly reacting sub-group A 3 whose agglutino-gen was not demonstrable with ordinary grouping sera, but was sufficient to cause an immune reaction in the mother.

The cases shown in detail in table IV are selected from a number of cases investigated, the total figures being given in table II and the findings in all heterospecific cases in table III.

TABLE II

*Changes in agglutination titre in mothers' serum in relation to type of pregnancy*

Type of pregnancy	Number of cases		
	Total	Agglutination titre	
		raised	not raised
Heterospecific . . .	46	40	6
Homospecific . . .	154	1	153
Total .	200	41	159

*Absence of iso-immunisation in heterospecific pregnancy*

It will be noted (table III) that in six cases of heterospecific pregnancy no rise in the maternal agglutinin was detected and an explanation for this difference in behaviour was sought. As regards the mechanism of production of the iso-agglutinin changes, it is suggested that the group-specific substances are absorbed from the foetus into the maternal circulation in a high proportion of cases. This might be due either to escape of foetal blood into the maternal circulation or to the passage of soluble substances across the placenta.

It has been shown by Schiff and Sasaki (1932), that in 75-80 per cent of the population group-specific substances are present in the tissue and body-fluids in demonstrable amounts, while in the other 20-2

per cent. these soluble substances are absent. Schiff showed that the two classes of individual could be readily recognised, the method depending upon demonstrating the presence or absence of A or B

TABLE III

*Cases of heterospecific pregnancy investigated*

No.	Blood group of mother	Rh	Agglutinin titre in early pregnancy		Agglutinin titre after delivery		Blood group of baby	Rh	Secretor or non-secretor
			Anti-A	Anti-B	Anti-A	Anti-B			
1	O	+	1:320	1:160	1:2560	1:320	A	+	S
2	O	+	1:640	1:80	1:640*	1:80*	A	+	N
3	O	+	1:80	1:20	1:5120	1:80	A	+	S
4	O	—	1:80	1:40	1:1280	1:160	A	+	S
5	O	—	1:320	1:20	1:320*	1:20*	A	+	N
6	O	—	1:40	1:80	1:80	1:2560	B	+	S
7	O	+	1:80	1:80	1:160	1:2560	B	+	S
8	O	+	1:40	1:160	1:80	1:5120	B	+	S
9	O	+	1:160	1:80	1:160	1:2560	B	+	S
10	O	+	1:80	1:40	1:80*	1:40*	B	+	N
11	O	+	1:160	1:40	1:1280	1:80	A	+	S
12	O	+	1:640	1:40	1:2560	1:80	A	+	S
13	O	+	1:80	1:160	1:160	1:1280	B	+	...
14	O	+	1:160	1:80	1:2560	1:320	A	+	...
15	O	—	1:320	1:80	1:1280	1:80	A	+	...
16	O	+	1:160	1:80	1:1280	1:320	A	+	...
17	O	+	1:160	1:80	1:640	1:80	A	+	...
18	O	+	1:320	1:320	1:320	1:1280	B	+	...
19	O	+	1:160	1:20	1:160	1:640	B	+	S
20	O	+	1:80	1:20	1:160	1:640	B	+	...
21	O	+	1:80	1:160	1:160	1:640	B	+	...
22	O	+	1:160	1:320	1:160	1:1280	B	+	...
23	O	+	1:320	1:320	1:320	1:8000	B	+	...
24	O	+	1:320	1:160	1:2560	1:160	A	+	...
25	O	+	1:320	1:80	1:2560	1:160	A	+	...
26	O	+	1:320	1:20	1:320*	1:20*	A	+	N
27	O	+	1:40	1:20	1:320	1:20	A	+	...
28	O	+	1:320	1:160	1:2560	1:160	A	+	...
29	A	—	...	1:20	...	1:640	AB	+	S
30	A	—	...	1:80	...	1:1280	AB	+	S
31	A	—	...	1:20	...	1:20*	B	+	N
32	A	—	...	1:160	...	1:1280	B	+	S
33	B	+	1:20	...	1:20*	...	A	+	N
34	B	+	1:160	...	1:640	...	AB	+	...
35	B	—	1:320	...	1:1280	...	A	+	S
36	B	+	1:160	...	1:640	...	A	+	...
37	B	+	1:320	...	1:2560	...	AB	+	S
38	B	+	1:160	...	1:2000	...	A	+	...
39	B	+	1:80	...	1:640	...	A	+	S
40	O	—	...	...	1:2560	1:40	A	+	S
41	O	+	1:20	1:20	1:40	1:640	B	+	...
42	O	+	1:20	1:40	1:320	1:40	A	+	...
43	O	+	...	...	1:320	1:40	A	+	...
44	O	+	...	...	1:12800	1:640	A	+	S
45	O	—	...	...	1:640	1:1280	B	+	...
46	A	+	...	1:20	...	1:640	B	+	...

\* No rise in titre.

substance in the saliva from its ability to inhibit anti-A or anti-B agglutinin in appropriate serum. Schiff noted that the concentration of group-specific substances was much greater in saliva than in serum



TABLE IV

*Details of examples of heterospecific and homospecific pregnancies*

Case no.	Age (years)	Blood groups		Agglutination titres in mother's serum			
		Mother	Baby	Relation to confinement		Anti-A	Anti-B
				Before	After		
6	20	O, Rh—	B, Rh+ Secretor	98 days		1:40	1:80
				77 "		1:40	1:80
				49 "		1:40	1:80
				21 "		1:80	1:2560
					2 days	1:80	1:2560
7	24	O, Rh+	B, Rh+ Secretor	126 days	35 "	1:80	1:640
					4 days	1:40	1:160
					21 "	1:160	1:2560
					350 "	1:160	1:640
						1:80	1:320
5	21	O, Rh—	A, Rh+ Non-secretor	84 days		1:320	1:20
					6 days	1:320	1:20
					364 "	1:320	1:20
10	36	O, Rh+	B, Rh+ Non-secretor	56 days		1:80	1:40
				28 "		1:80	1:40
					3 days	1:80	1:40
29	23	A, Rh—	B, Rh— Secretor	133 days		—	1:20
				27 "		—	1:160
					5 days	—	1:640
38	21	B, Rh+	A, Rh+ No secretor tests available		308 "	—	1:160
				91 days		1:160	—
				63 "		1:320	—
				56 "		1:320	—
					3 days	1:1280	—
7972	37	O, Rh+	O, Rh+		21 "	1:2560	—
				126 days		1:80	1:80
				112 "		1:80	1:80
				42 "		1:80	1:80
7871	20	B, Rh+	B, Rh+		4 days	1:80	1:80
				126 days		1:40	—
				105 "		1:40	—
				77 "		1:40	—
				56 "		1:40	—
7921	18	B, Rh+	O, Rh+	14 "		1:40	—
					3 days	1:40	—
				112 days		1:320	—
				63 "		1:320	—
					2 days	1:320	—
7884*	22	O, Rh+	O, Rh+		28 "	1:320	—
				196 days		1:320	1:320
				140 "		1:320	1:320
				105 "		1:640	1:320
				77 "		1:2560	1:320
				49 "		1:2560	1:320
					4 days	1:2560	1:640
7884*	22	O, Rh+	O, Rh+		35 "	1:1280	1:640
						1:1280	1:640

\* Exceptional case noted in the text.

and he appropriately named the two classes secretors and non-secretors. It will be observed that the proportion of mothers who failed to show iso-immunisation in the above group is approximately 13 per cent.; in such a small series this is sufficiently close to Schiff's 20 per cent. of non-secretors to make it worth while determining whether in fact the two groups coincided. Accordingly at a later date samples of saliva from a number of babies previously examined were tested to determine whether or not they were secretors.

*Technique.* The technique used is a modification of that described by Wiener (1943, p. 278). A sterile swab of absorbent cotton-wool is inserted below the infant's tongue and allowed to become thoroughly saturated with saliva. The swab is then soaked in 0.5 c.c. of isotonic saline for one hour at room temperature and heated for 20 minutes at 100° C. in a water bath to destroy the enzymes present. Finally the fluid is expressed from the swab and added to 0.5 c.c. of the test serum. The mixture of saliva and serum is allowed to remain at room temperature for two hours, after which the agglutinin titres are again assessed by the method previously described for maternal sera. Definite reduction in agglutinin titre indicates the presence in the saliva of A or B group specific substance, while, conversely, failure of the saliva to reduce agglutinin titre indicates their absence. A group O serum of suitable  $\alpha$  and  $\beta$  agglutinin titre was first used, but later when the supply of this serum failed a mixture of equal parts of anti A and anti B grouping was prepared. It is important that a serum mixture prepared in this way be left until a constant agglutinin titre is reached, as inhibition of  $\alpha$  and  $\beta$  agglutinin by A and B soluble group specific substances in the serum may occur. Control titrations of the serum mixture alone were carried out each time the mixture was used. The use of the mixture of  $\alpha$  and  $\beta$  agglutinins was convenient, as the amount of saliva available from infants is very small.

By this method, secretors and non-secretors may be distinguished. The results (table V) demonstrate that saliva obtained from the 18 infants whose mothers showed rising iso-agglutinin titres had a definitely inhibitory effect on the test serum, while samples from 6 infants whose mothers failed to show a rising titre during pregnancy (see tables II and III) had no such effect. Controls from group O individuals also failed to show any inhibitory action. These results may be taken as evidence that the infants who produced iso-immunisation in the mother were secretors, those not causing iso-immunisation non-secretors.

## DISCUSSION

From the foregoing results it is apparent that iso-immunisation of the mother in heterospecific pregnancy is very frequent and that a substantial rise in the corresponding iso-agglutinin titre is generally found. Since this phenomenon appears regularly when soluble group-specific substances are present in the infant's saliva and fails to appear when they are absent, it is suggested that iso-immunisation is normally brought about by the passage of soluble group-specific substances across the placenta into the maternal circulation, and

TABLE V—Results of titration of samples of the same test serum after two hours' absorption with saliva from babies

(Case numbers correspond with those of table III)

Case no.	Group of infant	Cells	Dilutions								Titre	Secretor or non-secretor
			1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		
1	A	A B	(+) ++	w (+)	— w	—	—	—	—	—	1:8α 1:16β	Secretor A
2	A	A B	v +	v (+)	++ w	+	+	(+)	w	—	1:256α 1:16β	Non-secretor
3	A	A B	++ ++	+	+	w	—	—	—	—	1:32α 1:16β	Secretor A
4	A	A B	w ++	(+)	— w	—	—	—	—	—	1:4α 1:16β	" A
5	A	A B	v ++	v (+)	++ w	+	+	(+)	w	—	1:256α 1:16β	Non-secretor
6	B	A B	v w	v	++	+	+	(+)	w	—	1:256α 1:4β	Secretor B
7	B	A B	v	v	++	+	+	(+)	w	—	1:256α Nil	" B
8	B	A B	v	v	++	+	+	(+)	w	—	1:256α Nil	" B
9	B	A B	v w	v	++	+	+	(+)	w	—	1:256α 1:4β	" B
10	B	A B	v ++	v (+)	++ w	+	+	(+)	w	—	1:256α 1:16β	Non-secretor
11	A	A B	+	(+)	w	—	—	—	—	—	1:16α 1:16β	Secretor A
12	A	A B	w ++	(+)	—	—	—	—	—	—	1:4α 1:8β	" A
19	B	A B	v	v	++	+	(+)	w	—	—	1:128α Nil	" B
26	A	A B	v ++	v (+)	++ w	+	+	(+)	w	—	1:256α 1:16β	Non-secretor
29	AB	A B	+	w	—	—	—	—	—	—	1:8α 1:4β	Secretor AB
30	AB	A B	w	—	—	—	—	—	—	—	1:4α Nil	" AB
31	B	A B	v ++	v (+)	++ w	+	+	(+)	w	—	1:256α 1:16β	Non-secretor
32	B	A B	v	v	++	+	+	(+)	—	—	1:128α Nil	Secretor B
33	A	A B	v ++	v (+)	++ w	+	+	(+)	w	—	1:256α 1:16β	Non-secretor
35	A	A B	++ ++	+	(+) w	w	—	—	—	—	1:32α 1:16β	Secretor A
37	AB	A B	++ w	+	w	—	—	—	—	—	1:16α 1:4β	" AB
39	A	A B	++ ++	++ (+)	+	(+)	w	—	—	—	1:64α 1:8β	" A
40	A	A B	w ++	(+)	w	—	—	—	—	—	1:4α 1:16β	" A
44	A	A B	+	(+)	w	—	—	—	—	—	1:16α 1:16β	" A
Untreated control	serum	A B	v ++	v (+)	++ w	+	+	(+)	w	—	1:256α 1:16β	...
Serum treated with group O saliva		A B	v ++	v (+)	++ w	+	+	(+)	w	—	1:256α 1:16β	...

v = visual agglutination in tube.

++ = coarse clumps microscopically.

+ = less coarse but obvious agglutinates.

(+) = small agglutinates of 5-7 cells.

w = small agglutinates of 3-5 cells

(taken as end-point).

? = indefinite, with loss of smoothness.

— = smooth, as in controls.

The titre (w) is expressed as the original dilution of serum before the cell suspension was added.

not, as has hitherto been supposed, by the leakage of foetal blood. It is therefore unnecessary to postulate defects in the placenta in explanation of the observed facts. Such defects may, however, occasionally occur and may be the explanation of the very high titres observed by Boorman, Dodd and Mollison (1944). In such cases, where enormous amounts of iso-agglutinin are formed, it may be supposed that the quantity of soluble group-specific substance in the foetal tissue fluids is insufficient to protect the foetus against the harmful antibody passing through the placenta from the maternal to the foetal circulation and, accordingly, that hæmolytic disease of the foetus may result from ABO incompatibility.

### SUMMARY

The iso-agglutinin titres of 200 primiparae were quantitatively assessed throughout pregnancy and the puerperium. Of these, 46 were heterospecific and 154 homospecific pregnancies. Forty of the heterospecific cases showed distinct evidence of iso-immunisation, exhibiting a rising agglutinin titre corresponding to the antigen in the child's blood. There was no evidence of any harmful effect upon the child in this series. The homospecific pregnancies, with one exception, failed to show any gross change in agglutinin titres during pregnancy.

The saliva from 24 of the children was tested for inhibitory powers against anti-A and anti-B sera, and were thus divided into secretors and non-secretors. The mothers who failed to show a rising titre, although the pregnancy was heterospecific, had non-secretor children, while the children shown to be secretors produced iso-immunisation of the mother. The suggestion is therefore made that iso-immunisation is usually due to absorption by the mother of group-specific substances from the foetus *in utero*.

I should like to thank Professor D. F. Cappell for his very valuable suggestions, advice and criticism during the progress of this investigation. I am indebted to Professor Margaret Fairlie for access to the clinical material and to the staff of the Maternity Hospital, Royal Infirmary, Dundee, for their helpful co-operation. To Dr Marjory N. McFarlane my thanks are due for the Rh determinations and for help in grouping cord blood samples.

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# A NON-HÆMOLYTIC GROUP K STREPTOCOCCUS FROM A CASE OF ENDOCARDITIS

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THE organism here described was isolated from the blood stream of a case of bacterial endocarditis; because of certain anomalous characteristics it was fully investigated and found to be a non-hæmolytic streptococcus belonging to Lancefield's group K.

## Case history

The patient (Ll-H), a farmer aged 50 years, gave a history of 6 weeks malaise and fever and of left-sided abdominal pain for 2 weeks before admission to hospital. His appetite had been poor and he had lost some weight. There was no previous history of heart disease or of any other relevant illness.

On examination he was pyrexial, but physical signs were few and inconclusive. During the 10 weeks he was under observation, however, the full clinical picture of subacute bacterial endocarditis developed, with varying organic cardiac murmurs, splenomegaly, cutaneous petechiæ and hæmaturia. A non-hæmolytic streptococcus was isolated by blood-culture on eight occasions over a period of 52 days. On the first occasion no colony count was made, but from the later cultures 13, 7, 22, 13, 34, 7 and 8 colonies per c.c. were obtained.

Intensive treatment with sulphathiazole and sulphadiazine was unsuccessful and penicillin was not available. He was discharged home two weeks before death and a post-mortem examination was resolutely refused by the relatives. No primary focus of infection was discovered and there was no evidence of any congenital or previously acquired heart disease. The Kahn test was negative. That the organism was causally connected with the patient's illness is suggested not only by its repeated isolation from the blood stream, but also by the presence in the serum of specific agglutinins to a titre of 1:10,000. The streptococcus was not agglutinated by normal serum in dilutions of 1:25 to 1:250.

## Bacteriological findings

The organism was first regarded as an enterococcus, but as it failed in many respects to conform to the accepted criteria (Sherman, 1938; Ehrismann, 1943; Rantz and Kirby, 1943) of the *faecalis-lactis*

group, it was examined serologically and found to be a group K strain.' This was confirmed in a second laboratory with grouping serum from a different source.

The cultural and biochemical properties of the strain were therefore examined and compared with Hare's (1935) description of hæmolytic group K strains, and with the *faecalis-lactis* streptococci.

*Morphology.* The organisms are small cocci about  $0.5\ \mu$  in diameter, growing in broth in pairs, small clumps and short chains. When paired they tend to be lanceolate. Both chain formation and the lanceolate shape become less frequent after many subcultures. No capsule is detectable in young (4-6 hour-) or older cultures. The cocci are non-motile, Gram-positive and non-acid-fast.

*Growth requirements.* Growth is good on simple media and is not improved by the addition of blood or serum. Optimum temperature is  $37^{\circ}\text{C}$ .; growth is negligible at room temperature and very poor at  $45^{\circ}\text{C}$ . The organism is an aerobe, and a facultative anaerobe; growth is not affected by increased  $\text{CO}_2$  tension.

*Cultural characteristics.* On agar the colonies are small, slightly opaque and colourless, with a wavy edge and fine striations radiating outwards from a slightly raised central cone. After 18 hours the colonies are barely visible to the naked eye, but after 4 or 5 days they attain a diameter of up to 2 mm. No hæmolysis occurs on sheep or horse blood agar aerobically at  $37^{\circ}\text{C}$ . or at room temperature, or anaerobically. When first isolated cultures in broth and serum broth showed an easily dispersable finely granular deposit, with a faintly turbid supernatant. After a few subcultures a diffuse turbidity above a similar deposit appeared and has since remained a constant characteristic. In 0.2 per cent. glucose broth there is turbidity with a heavy flocculent deposit, and floccules adhering to the side of the tube which are easily detachable by light shaking. On MacConkey agar the organism grows as small red pin-point colonies. On one batch, made with inferior peptone, no growth was obtained. In gelatin there is no growth at room temperature and growth but no liquefaction at  $37^{\circ}\text{C}$ . Shake cultures in glucose agar show throughout the medium lenticular colonies up to 1 mm. diameter in 48 hours.

The cocci are killed in less than 10 minutes at  $60^{\circ}\text{C}$ . and survive up to 3 months on agar at room temperature.

*Biochemical reactions.* Acid but no gas is produced in aesculin, dextrin, fructose, galactose, glucose, glycogen, inulin, lactose, maltose, mannitol, mannose, raffinose, salicin, starch, sucrose and trehalose. There is no fermentation of amygdalin, arabinose, dulcitol, erythritol, inositol, rhamnose, sorbitol and xylose. Litmus milk is acidified, clotted (acid type) and slightly decolourised. Voges-Proskauer test is negative, methyl red test positive. Indole is not formed. There is no hydrolysis of sodium hippurate: the pH attained in 1 per cent. glucose broth is 4.2: there is no reduction of 0.1 per cent. methylene blue in milk. The organism is not bile-soluble. Growth is slight in

10 per cent. and absent in 40 per cent. bile salt broth and very poor in 6.5 per cent. NaCl broth. There is no growth at pH 9.6. S— and 0— haemolysin, fibrinolysin, catalase and coagulase are not produced.

**Drug resistance.** There was no inhibition of growth in *p*-amino-benzoic acid-free broth containing 100 mg. of sodium sulphathiazole per 100 c.c., or in a ditch plate preparation. Using home-made crude penicillin, the organism appeared slightly less sensitive than the Oxford staphylococcus by the agar cup technique and was inhibited up to 1:32 dilution of the filtrate as compared with 1:64 for the staphylococcus.

*Pathogenicity.* 0.5 c.c. of an 18-hour broth culture failed to kill a mouse after intraperitoneal inoculation.

The characteristics of this streptococcus, of Hare's group K streptococci and of the *faecalis-lactis* group are compared in the accompanying table.

TABLE

#### Comparison of strain LL-H, group K and faecalis-lactis streptococci

	Strain LI-H	Group K (flare)	<i>Faecalis-lactis</i> group
Chain formation . . . . .	±	...	—
Hæmolysis on blood agar . . . . .	—	+	±
Growth in 40 per cent. bile broth . . . . .	—	—	+
"    " 6.5 per cent. NaCl . . . . .	—	...	+
"    " at pH 9.6 . . . . .	—	...	+
"    " 45° C. . . . .	—	...	+
Heat resistance . . . . .	—	—	+
Reduction of methylene blue . . . . .	—	—	+
. . . . .	4.2	5.2-5.4	4.2-4.7
. . . . .	—	—	—
. . . . .	+	...	+
. . . . .	+	...	—
Lactose . . . . .	+	+	+
Mannitol . . . . .	+	—	±
Raffinose . . . . .	+	...	—
Salicin . . . . .	+	...	±
Sorbitol . . . . .	—	—	+
Sucrose . . . . .	+	...	+
Trehalose . . . . .	+	+	—

± indicates that hæmolysis, fermentation of the sugar or chain formation by strains within the group is inconstant.

\* One strain in 8 fermented trehalose.

## Discussion

It is evident from these data that the coccus resembles Hare's group K strains much more closely than it does the *faecalis-lactis* group. According to both Sherman and Ehrismann the most reliable criteria of enterococci are their ability to grow over a wide range of temperatures, at a pH of 9.6 and in 6.5 per cent. NaCl broth. Rantz and Kirby consider good growth in 6.5 per cent. NaCl broth and in 0.1 per cent. methylene blue in milk adequate criteria. The coccus



fulfils none of these, nor does it resemble the  $\alpha$ -hæmolytic strains of bovine origin. It differs from the eight strains described by Hare in the pH produced in glucose broth, in its fermentation of mannitol and in the absence of hæmolysis on blood agar. The biochemical activity of group K streptococci, however, cannot be regarded as well defined on the basis of a study of eight strains, but the lack of hæmolytic action raises questions of considerable importance.

Apart from variability of hæmolysis by group D streptococci, which include about 50 per cent. of all *fæcalis-lactis* strains (Rantz and Kirby, 1943; Lancefield, 1940-41—according to Sherman *all* the enterococci except *Str. lactis*), it is becoming increasingly recognised that non-hæmolytic strains and variants do occur in most of Lancefield's groups. Noël (1934 *a* and *b*) claimed to have converted hæmolytic streptococci into "enterococci" by culture in increasing concentrations of urine, but his protocols are inadequate and hardly convincing. Todd (1928), however, had already observed the occurrence during animal passage of non-hæmolytic variants in two strains of  $\beta$ -hæmolytic streptococci from cases of puerperal sepsis, but he was able to show that under anaerobic conditions hæmolysin was still formed. Later (1934) he showed that the oxygen-labile streptolysin is peculiar to group A strains. More recently Coburn and Pauli (1941) described an epidemic spread by carriers of non-hæmolytic group A streptococci and found that hæmolysis might occur only during growth at 22° C., while Colebrook *et al.* (1942) have described an outbreak of wound infection due to non-hæmolytic group A strains. Out of the 13 strains isolated none produced soluble hæmolysin, but 11 (all type 12) showed  $\alpha$ -hæmolysis on a different batch of medium, and 9 of these and one other produced oxygen-labile hæmolysin anaerobically. This phenomenon was not related to the green variation described by Fry (1933) and Fuller and Maxted (1939).

Among group B strains hæmolytic activity is very variable (Stableforth, 1932; Lancefield, 1934). In group C non-hæmolytic strains have apparently not yet been described. Among the remaining groups "certain non-hæmolytic or doubtfully hæmolytic strains are sometimes encountered, particularly in groups H and K" (Lancefield, 1940-41), but no other reference to such group K strains has been found.

The strains originally described by Hare were derived from routine throat swabs and were not considered to play any pathogenic role in the patients from whom they were isolated. Wheeler and Foley (1943), however, in a study of "several thousand strains" of hæmolytic streptococci, found group K strains in four cases of sinusitis and in one of brain abscess. Of the cases of sinusitis three were members of the same family; in the fourth the organism was present in pure culture. All five patients recovered, and Wheeler and Foley did not consider that the ætiological significance of the strains was clearly established. The importance of the present case, therefore, lies not

only in the further demonstration that hæmolysis is not a safe primary criterion for classifying streptococci (as Colebrook remarks, we badly need some other pointer), but also in showing that a group K streptococcus may have sufficient virulence to produce bacteriæmia and endocarditis in man with fatal results. Moreover, it is possible that many non-hæmolytic strains now generally dismissed as probable members of the *faecalis-lactis* group will be found serologically to belong to Lancefield's groups. The recent isolation, during the writing of this note, of another non-hæmolytic group K strain—the predominating organism in pus from a cerebral abscess secondary to frontal sinusitis, though its ætiological significance was not established—lends weight to the belief that a constant lookout for such strains might well be rewarded, and as serology is a more reliable guide than hæmolysis from an epidemiological point of view—and possibly therapeutically—the question may be of more than academic importance.

### Summary

A non-hæmolytic group K streptococcus isolated from a case of subacute bacterial endocarditis is described. Comparison is made between this organism, hæmolytic group K strains and non-hæmolytic streptococci of the *faecalis-lactis* group. The implications of the findings are discussed.

The media used were supplied by the London County Council Southern Group Laboratory, with the exception of certain sugars which were made up in L.C.C. peptone water and the nutrient agar on which the absence of hæmolysis was confirmed. L.C.C. digest broth was used as the base for the various special broth media.

Thanks are due to Professor S. P. Bedson and Dr C. F. Barwell for help and advice, to the staff of the Veterinary Department of the University College of North Wales and to Dr Dora Colebrook for grouping the streptococcus, and to Dr Emyr Jones, Medical Superintendent, County Hospital, Bangor, for information on the clinical aspects of the case.

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## THE LIFE-SPAN OF THE RED CELL IN MAN

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THE methods which have been applied to the determination of the life-span of red cells have so far given altogether discordant results and the rationale of every one of them is of more or less questionable validity. Many bibliographies of the subject are available; Schiødt (1938) gives a useful collection of references which, though neither exhaustive nor up-to-date, exemplifies shortly the various techniques and numerical results. Our object in this paper is to present data on the course of destruction of erythrocytes after transfusion into normal subjects, as determined by the differential agglutination method of Ashby (1919). We shall show that from them, under certain assumptions, it is possible to calculate not merely the average life of the cells as they occur naturally, but also their chance of survival for any length of time.

### *Survival of transfused red cells in normal subjects*

A considerable body of data on the survival of transfused cells in a pathological environment is now available; about thirty cases have been followed in these laboratories (for some of these see Brown *et al.*, 1944). To them may be added about ten others described in the literature in sufficient detail for quantitative examination. In the paper referred to it was shown that at least two mechanisms of cell destruction might be distinguished and that one of them appeared to be strikingly regular in its action. In many cases the curve connecting the number of cells surviving with time from transfusion was approximately linear over the latter part of its length, and, except in some clinically severe ones, its end-point lay near 100 days. This result has been repeatedly obtained by other workers. It was tentatively concluded that in normal circumstances the curve would be linear throughout and that the regularities observed depended on a property of the transfused cells themselves. The cases which have since been investigated have all given results in agreement with previous experience.

We now suggest that the linear curve is in fact merely the expression of the erythrocytes being destroyed in the recipient by normal means.

so as to remove in unit time a fixed fraction of those introduced at transfusion, and we take as the value of this fraction the mean of  $L_s$  found above, namely 0.0083, i.e. 0.83 per cent. per day.

The data from these three subjects and also from six hypochromic anæmias previously investigated were further submitted to Jeffreys' (1939) K-ratio test in order to confirm if possible the hypothesis that the decay curve is essentially linear (see appendix). For this purpose

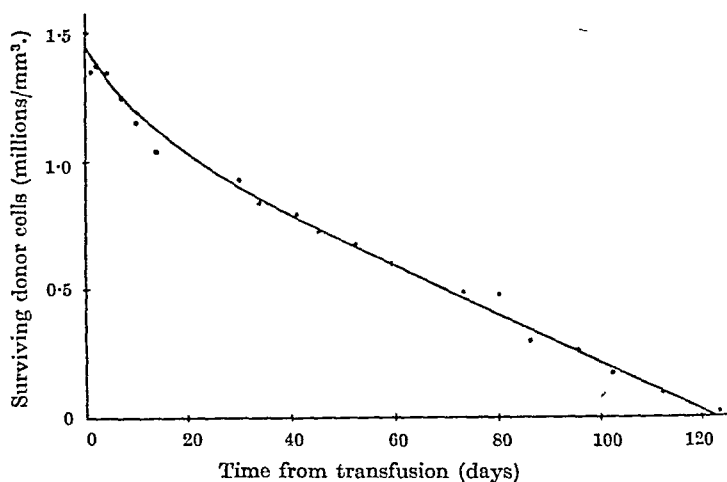


FIG. 2.—Survival of transfused erythrocytes in a normal recipient (subject III).

a linear equation was fitted only to those points in each graph for which  $t$  was more than 25 days, so as to avoid disturbances following transfusion.

Number of graphs undergoing test	.	.	.	.	9
Total number of point data involved	.	.	.	.	108
Total number of changes of sign	.	.	.	.	54
Number of parameters determined from each graph	.	.	.	.	2

$$\chi^2 = 1.0$$

From Jeffreys' table,  $K > 1$ .

The hypothesis of linearity is thus in accord with the available evidence.

### *Expectation of life of the red cell*

In this section we deal first with the relation between the observed law of decay of a body of transfused cells and the law of survival of the individual erythrocyte in an idealised case. The relation is expressed in a perfectly general form which necessarily holds if the assumptions involved are valid. The extent to which they may be fulfilled in practice is discussed below. The necessity for a mathematical treatment arises because transfused blood contains

cells of every possible age; the rate of destruction in the recipient depends on the effective viability of all these simultaneously. Thus the law of survival of the individuals, as a function of their own age and not of the time from transfusion, is not directly apparent.

Let  $\phi(\tau)$  be the chance that a cell newly released into circulation will live to an age  $\tau$  at least; i.e. of a number of cells released simultaneously, a fraction  $\phi(\tau)$  will still be present after time  $\tau$  from their "birth". If cells are being introduced into the blood stream at a constant rate  $n$  per unit volume per unit time, there will always be present  $n\phi(\tau)d\tau$  cells (per unit volume) which are of an age between  $\tau$  and  $\tau+d\tau$ . Altogether there are

$$n \int_0^{\infty} \phi(\tau) d\tau$$

cells of all ages, and this is the total count, say  $N_1$ . Hence from a knowledge of  $N_1$  and  $\phi(\tau)$ ,  $n$  may be deduced, and the true average life of the cells is  $N_1/n$ .

Now suppose that some of these cells are removed to another similar system from whose proper cells they are distinguishable, so that after dilution they are present in number  $N_0$  per unit volume. The transfused cells are of all ages, the function giving their age distribution being from the above  $n\phi(\tau)d\tau/N_1$ . Let time ( $t$ ) be now reckoned from the time of transfusion. At time  $t$  in the new era there are present a fraction

$$n\phi(t+\tau)d\tau/N_1$$

of cells which were of age between  $\tau$  and  $\tau+d\tau$  at  $t=0$ . So the total number of cells surviving is

$$N = N_0 \frac{n}{N_1} \int_0^{\infty} \phi(\tau+t) d\tau = N_0 \frac{n}{N_1} \int_t^{\infty} \phi(\tau) d\tau.$$

This, then, is the relation between surviving donor cells and time which is actually measured in our experiments. Let the experimentally determined law be represented by

$$N = N_0 \psi(t).$$

Then we should have

$$\psi(t) = \frac{n}{N_1} \int_t^{\infty} \phi(\tau) d\tau$$

whence, by differentiation, finally

$$\phi(\tau) = -\frac{N_1}{n} \frac{d}{d\tau} \left\{ \psi(\tau) \right\}.$$

Applying the experimental results we have for the law of decay of transfused cells

$$N = N_0(1-L_e t), \text{ i.e. } \psi(t) = 1-L_e t \quad (t < 1/L_e) \\ = 0 \quad (t > 1/L_e).$$

Hence for  $\tau < 1/L_s$

$$\phi(\tau) = -\frac{N_1}{n} \frac{d}{d\tau} \left\{ \psi(\tau) \right\} = \frac{N_1 L_s}{n}.$$

Now  $N_1 L_s/n$  is constant, and since  $\phi(0)$  is necessarily equal to 1,

$$\begin{aligned} \phi(\tau) &= 1 & (\tau < 1/L_s) \\ &= 0 & (\tau > 1/L_s). \end{aligned}$$

That is to say, all the red cells live for a fixed time  $\bar{\tau} = 1/L_s$ , which is the true average life, and are then destroyed. On account of the variability of biological phenomena, there will in practice of course be some scatter about  $\bar{\tau}$ , but it is small, and our experiments are not exact enough to determine it. So the law of survival will take the form of fig. 3*b*, leading to fig. 3*a* for the destruction of transfused cells. Or otherwise: the chance of a cell's being destroyed at age

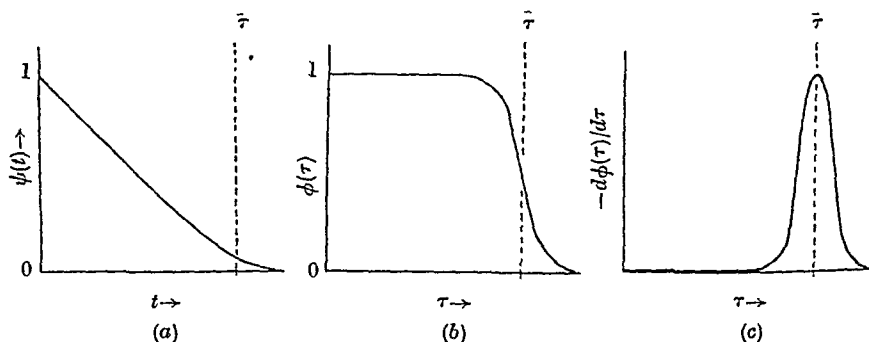


FIG. 3.—The ordinates are proportional to

- (a) the number of cells of transfused blood surviving from a time  $t$  after transfusion;
- (b) the chance that a cell will "live" for a time  $\tau$  from its release into the blood stream;
- (c) the chance that a cell will be destroyed at a time  $\tau$  after its release into the blood stream.

$\tau$  is proportional to  $-d\phi(\tau)/d\tau$ , which is the death-rate (fig. 3*c*); most cells are destroyed within a few days of the average life—rarely much before or much after. This is the result adumbrated by Schiødt (1938).

The value of  $L_s$  has been determined above to be  $0.0083 \text{ day}^{-1}$ . Hence the true average life of the erythrocyte is about 120 days. Taking the total red cell count ( $N_1$ ) in normal males as  $5.4$  millions/ $\text{mm}^3$ , the mean rate of formation (and destruction) is given by

$$\frac{N_1 L_s}{n} = 1; \quad n = 5.4 \times 10^6 \times 0.0083.$$

This works out at 45,000 cells per  $\text{mm}^3$ . per day, corresponding to a daily total production of about a quarter of a billion ( $2.5 \times 10^{11}$ ) cells.

*Agglutinability of young cells*

In order to meet a criticism of the Ashby method which is mentioned below, the following experiment was carried out.

In a patient suffering from pernicious anaemia and responding to liver therapy the red cell count was 2.56 millions/mm<sup>3</sup>, of which 13.4 per cent. were reticulocytes. The inagglutinable count, determined in the usual way, was 42,000/mm<sup>3</sup>. The inagglutinable count was then re-estimated, the cell dilution being made with citrate solution containing three drops of cresyl blue stain in 1 c.c. It was found to be 38,000/mm<sup>3</sup>. The slide showed that 15.0 per cent. of the unagglutinated cells were reticulocytes. The differences are insignificant; the proportion of immune cells is the same among reticulocytes as among mature cells.

*Discussion*

Three principal assumptions are made in the above treatment. (a) That the rate of production of cells is constant, so that the age distribution in donor blood depends only on  $\phi(\tau)$ . While this is not strictly true, it is more nearly so in our particular experiments, in which mixed bloods were used, than if the blood had been derived from a single donor on one occasion. (b) That the blood volume remains constant in the recipient during the time of the experiment. Actually an adjustment of fluid volume takes place during and for a short time after transfusion; only the first few counts are affected. Subsequent changes are no doubt responsible for irregular counts, but these are smoothed out in the final results, whose close agreement justifies our neglecting minor blood volume changes. (c) That the circumstances attending transfusion—storage and concentration of donor blood, special properties of the recipient's circulatory system—do not affect the donor cells in respect of their viability. This cannot be proved from internal evidence alone; in the present experiments we have tried simply to give the transfused cells an environment as nearly normal as the technique will allow. It should be noted here that survival experiments have been carried out in which cells were identified by their MN or Rh agglutinin reactions (*e.g.* Mollison, 1943); substantially similar results are obtained whatever system is used. It is unlikely that there is any gross error in the identification and measurement of transfused cells by the Ashby method.

The figures we have obtained here for the average life of transfused cells are on the whole somewhat higher than those found (mean 53 days) in hypochromic anaemia, the most nearly normal condition previously examined. The difference is statistically significant by the *t* test (probability of a greater divergence less than 0.02). The influence of sex has not been considered; our three normal subjects were men, while of the six hypochromic anaemias,



five were women. Since  $N_1 = n\bar{\tau}$ , and since  $N_1$  is lower in women than in men, in the former the rate of blood formation must be slower or the average life of the cell shorter; in any case loss at menstruation will cause some reduction in  $\bar{\tau}$ . Now in women  $N_1$  is about 4.8, in men about 5.4 millions/mm<sup>3</sup>. The ratio is 0.89. Remembering that for a linear decay curve  $\bar{\tau} = 2\bar{l}$ , the ratio of the average lives as indicated by the hypochromic anæmias and by the normal subjects is  $106/120 = 0.88$ . This shows that the discrepancy can be sufficiently accounted for by the difference in sex even if no pathological factors are operative, though it is too early to attach any further weight to this result.

Of the methods employed for the determination of the average life, those which depend on the estimation of the amount of iron or pigment excreted (*e.g.* Heilmeyer, 1932; Bingold, 1930, quoted by Schiødt, 1938) suffer from the defect that the amount which is reabsorbed after breakdown to form new cells is quite uncertain; the figures given vary from 20 to 200 days. Those which depend on the time of recovery from an excess or deficit of cells brought about artificially (*e.g.* Barcroft *et al.*, 1923; Schiødt, 1937) neglect the fact that both blood formation and blood destruction must be acting under constraint, and the results, usually near 30 days, are almost certainly too low—the organism tends to alter its behaviour in such a way as to hasten its return to normality—while also the end-point of the recovery period is difficult to determine because it is approached slowly and becomes obscured by fluctuations in the red cell count due to outside causes.

The method of differential agglutination has the advantage that the cells involved were formed in the natural way, and only their breakdown can occur in any unusual manner. It is improbable that the destructive agencies would especially favour the survival of the transfused cells; if this is so, the figure we have obtained for the average life is unlikely to be too great. We know of no more positive evidence which conflicts with our findings or conclusions. Rous (1923) suggested that that part of the stroma which carries (or fails to carry) the agglutinogens is not completely destroyed at the cell's breakdown, but is partly re-utilised in the formation of new ones. Thus some of the recipient's own cells, formed after transfusion, will come to possess immunity similar to the donor's, and the estimated survival time will be greater than the true value. It can readily be seen, however, that if this took place to any extent, according to a simple law, it would result in a decay curve of roughly exponential shape; complicated and quite *ad hoc* hypotheses would have to be invoked to account for the linear curve actually found. Again (Isaacs, 1924) it has been thought that the inagglutinable counts are vitiated by a burst of erythropoiesis occurring at transfusion, the young cells formed being for a time devoid of agglutino-gen. This idea does not seem to be based on any evidence. If the phenomenon occurs, it is

difficult to see how it could do more than disturb the apparent course of destruction during a short initial period. Our experiment (p. 135 above) seems to indicate that it does not happen.

Perhaps the most promising alternative method of determining the average life is, in principle, to measure the length of time for which the red cell is a reticulocyte; then, if the proportion of reticulocytes among all the red cells leaving the marrow can be found, the average life can be calculated from the percentage normally in circulation. Some progress in this direction has already been made (Heilmeyer and Westhäuser, 1932; Baar and Lloyd, 1943*a*). Unfortunately there is complete disagreement as to the fraction of reticulocytes among newly-formed cells. Heath and Daland (1930) thought that most were fully matured before leaving the marrow, Baar and Lloyd (1943*a*) that none was. The latter authors deduced an average life of 42 days; in view of their recent criticism of the Ashby method (Baar and Lloyd, 1943*b*) it should be recognised that this result depends as much on their unproved assumption as on the experimental data.

If more cogent evidence should be brought to show that our conclusions are incorrect, it will mean that the organism does not treat foreign cells in the same way as native ones, and the existence of the relation deduced above between the decay of transfused cells and the law of survival of the individual erythrocyte will enable the aberration to be described quantitatively and perhaps lead to an explanation.

### Summary

1. The survival of transfused erythrocytes has been followed in three normal male subjects of group A, from whom blood was removed and replaced by blood of group O.

2. The curve of decay of the transfused cells was linear within the experimental error; their average life was 60 days.

3. The relation between the decay of transfused cells and the law of survival of the individual erythrocyte has been deduced.

4. It is concluded that red cells all live for approximately the same time, 120 days in men, which is the true average life. The normal rate of replacement is 0.83 per cent. per day.

5. Other methods of determining the average life are discussed and compared.

We are indebted to Dr G. L. Taylor of the Galton Laboratory, Cambridge, for supplies of sera, and to Miss Sheila Habgood and Miss Brigitte Wolff for technical assistance.

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### Appendix

#### THE APPLICATION OF JEFFREYS' K-RATIO AS A CRITERION FOR CURVE FITTING

In some biological investigations, especially those involving human subjects, it is not always possible to maintain constant conditions throughout a series of experiments of the same type. Where the object of the investigation is to describe quantitatively the behaviour of one variable (say  $y$ ) as a function of another independent variable (say  $x$ ), special difficulties may arise unless other variables are held constant. Hypothesis may have indicated the form of the unknown function expressing  $y$  in terms of  $x$ , but the sets of measured values of  $y$  and  $x$  from different experiments will not be superposable if the parameters or "constants" of the function differ in each (the parameters represent the mode of entry into the  $x$ - $y$  relation of variables not studied). Thus, for instance, there may be reason to suppose that the relation between  $y$  and  $x$  is always linear :

$$y = ax + b,$$

but if the  $a$  and  $b$  differ in each experiment, the true value of  $y$  corresponding to a given  $x$  will also differ.

As a test of goodness of fit for curves fitted to data of this class, Jeffreys' K-ratio is especially suitable and easy in application. If the fitted curve is a true representation of the underlying law, the deviations of  $y$  from the curve may be assumed to be random; each is as likely to be positive as negative. The chance of a change of sign of the error on passing from one point to the next is thus  $\frac{1}{2}$ . To test the adequacy of fit of the curve we have therefore to compare the number of changes of sign actually found with that to be expected if the errors are random, for a run of errors of the same sign is indicative of a systematic departure of the curve from the data. If we write  $q$  for the chance of a change of sign, then Jeffreys' K is, roughly speaking,

$$\frac{\text{Probability that } q = \frac{1}{2}}{\text{Probability that } q \neq \frac{1}{2}}.$$

In this particular application, where a number of curves of the same type are to be tested simultaneously,

$$K = \left[ \frac{2\{\Sigma p - N(m+1)\}}{\pi} \right]^{\frac{1}{2}} \exp \left[ -\frac{1}{2} \frac{\{\Sigma p - 2\Sigma c + N(m-1)\}^2}{\Sigma p - N(m+1)} \right]$$

in which  $N$  = number of curves

$\Sigma p$  = total number of point data

$\Sigma c$  = total number of changes of sign of deviations of  $y$

$m$  = number of constants in the equation to the curve determined from the data.

Jeffreys' table of  $K$  is entered in exactly the same way as the usual  $\chi^2$  table :

Jeffreys'  $n = \Sigma p - N(m+1)$

Jeffreys'  $\chi^2 = \frac{\{\Sigma p - 2\Sigma c + N(m-1)\}^2}{\Sigma p - N(m+1)}$ .

If  $\chi^2$ , for this  $n$ , corresponds to a  $K$  greater than 1, the random hypothesis is supported. The type of curve fitted to the data can then be regarded as satisfactory, and it need not be rejected if  $K$  is greater than  $10^{-1}$ . As usual, this only means that an increase in the complexity of the equation is not warranted by the accuracy of the data. If  $\chi^2$  corresponds to a  $K$  less than  $10^{-1}$  or  $10^{-1}$  (according to the computer's judgment), a refinement may be considered.



## SHORT ARTICLES

612 . 392 . 015 (B+C) : 612 . 018 . 65]—06 : 612 . 621 . 1 . 062  
(*Rattus*)

### THE INTERRELATIONSHIP BETWEEN VITAMINS B AND C AND THE FEMALE SEX HORMONES IN THEIR ACTION ON THE SEX ORGANS OF THE OVARIECTOMISED RAT

V. KORENCHEVSKY and K. HALL

*From the Lister Institute, London*

(PLATE XVII)

In previous papers we have studied the interrelationship between thyroid hormone and vitamins. This paper describes the effects on the uterus and vagina of extra amounts of vitamins B and C administered together with female sex hormones. There are only a few papers relating to the subject. Biskind and Glick (1936) and Ley (1937*a* and *b*) found that ovaries contain considerable amounts of ascorbic acid. According to Biskind and Glick, in non-pregnant cows the corpora lutea contain 0.3-1.4 mg. of ascorbic acid per g. of tissue, and the greater the development of corpora lutea the higher is the vitamin C content. In pregnant cows the corpora lutea contain 1.5-2.2 mg. In human ovaries Ley has obtained the following figures in mg. per 100 g.:—11.9 in ovaries without ripe follicles and corpora lutea, 23.8 in ripe follicle, 34.5 in corpora lutea menstruationis, 84.4 in corpora lutea graviditatis and 142.7 in corpora lutea graviditatis after administration to the patient of 300 mg. of vitamin C on the day before ovariectomy. From all these data Ley concluded that the more active the ovaries the greater is their vitamin C content and their need for it. He therefore treated 10 pregnant women who had previously suffered from habitual abortion, and, as he expected, was able to prevent this.

Israel and Meranze (1941) attempted to discover the significance of vitamin C for pregnancy by means of experiments on infantile normal and ovariectomised rabbits and ovariectomised mice and rats. They divided these animals into 3 groups treated with (a) oestradiol only, (b) oestradiol and vitamin C and (c) oestradiol and progesterone. They obtained more conclusive results in rabbits than in mice or rats. Histologically oestradiol+vitamin C produced progestational changes similar to but quantitatively less pronounced than those produced by oestradiol+progesterone. They therefore concluded that vitamin C has a progestational property and function.

#### TECHNIQUE

Two experiments were performed on 31 ovariectomised rats, but since the same results were obtained in both experiments, the data were pooled. The arrangement of the experiment into 6 groups, the number of rats in each group and the average figures obtained are given in the table. The average final age of the rats was 156 days and the duration of the experiment was 28 days.

Treatment was as follows. Oestradiol benzoate-butyrate (8  $\gamma$ ) was injected into all rats during the first week only, 3 times in the first experiment and

twice in the second experiment. From the second week the rats of groups I-IV were injected subcutaneously with 0.2 c.c. of pure oil, those of groups V and VI with 2 mg. of progesterone dissolved in 0.2 c.c. of oil, in each case 6 times a week.

TABLE

*Effect of the simultaneous administration of vitamins B and C with female sex hormones on the uterus and vagina of ovariectomised rats*

	Rats injected with oestradiol benzoate-butyrate					
	alone	and vitamins B	and vitamin C	and vitamins B+C	and progesterone	and progesterone and vitamins B+C
1. Uterus (wt. in mg.) .	204	234	208	222	199	203
2. Vagina (wt. in mg.) .	319	325	285	346	343	329
3. Final body weight (g.)	275	261	264	238	282	271
4. Epithelial metaplasia in uterus	5+	1+	4-	1+	5-	5-
5. Progestation-like changes in uterus	8-	4-	1+	3+	5+	5+
6. Changes in vagina .	Œstrous	Œstrous	Œstrous	Œstrous	Progestational	Progestational
7. No. of rats in group .	8	4	4	5	5	5
8. No. of group .	I	II	III	IV	V	VI

In lines 4 and 5, + = presence, - = absence of metaplasia or progestation-like changes. The number of rats in each group showing these changes is given before the + or - sign.

Vitamins B were given as follows. Rats of groups II, IV and VI received by mouth, 6 times a week, aneurin 0.48 mg., riboflavin 0.48 mg. and nicotinic acid 4.2 mg., and in addition, 3 times a week, subcutaneous injections of aneurin 0.56 mg., pyridoxin 0.56 mg. and calcium pantothenate 1.6 mg.

Groups III, IV and VI received, 6 times a week, 24 mg. of ascorbic acid by mouth and 2 or 3 times a week 25-30 mg. by subcutaneous injection.

It is necessary to emphasise that the rats were fed on our usual diet, which already contained an optimal amount of all vitamins. Therefore in these experiments the effects of large additional amounts of vitamins B and C were studied.

#### EXPERIMENTAL RESULTS

This paper describes only a histological investigation of the uterus and vagina. Other organs will be examined later. Sections were stained with iron hæmatoxylin and eosin.

#### Vagina

The weight and histological structure of the vagina showed no difference in groups II-IV as compared with group I, or in group VI as compared with group V. In groups I-IV keratinisation of the vaginal epithelium showed a typical œstrogenic effect, while in groups V and VI the epithelial layer was mucified (typical progestational effect).

Therefore in the case of the vagina vitamins B and C did not affect the œstrous changes produced by œstradiol or the progestational effect of combined treatment with progesterone and œstradiol.

#### Uterus

On the other hand, extra amounts of vitamins administered to rats injected with œstradiol (groups II-IV) produced the following changes in the uterus.

## EFFECT OF VITAMINS B AND C AND FEMALE SEX HORMONES ON UTERUS OF OVARIECTOMISED RAT

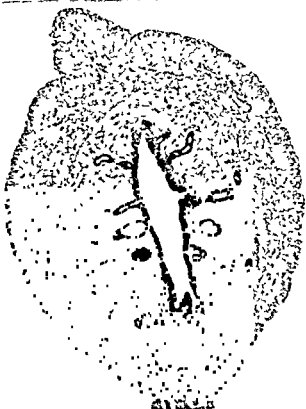


FIG. 1.—Ovariectomised rat injected with 8  $\gamma$  oestradiol benzoate-butyrate 3 times only during first week of experiment. Lumen without mucosal foldings, high epithelium. Approximately in middle of section, squamous metaplasia in four mucosal glands, in two on left of uterine lumen completely, and in the others (right of uterine lumen) partially closing the lumen of the glands. Rat 256 g., uterus 153 mg.



FIG. 2.—Ovariectomised rat injected during first week with oestradiol benzoate-butyrate (as in fig. 1) and during 4 following weeks with 2 mg. progesterone daily 6 times a week. Typical progestational changes—foldings of mucosa, low columnar epithelium, uneven enlarged lumen. Rat 260 g., uterus 203 mg.



FIG. 3.—Ovariectomised rat injected with oestradiol benzoate-butyrate as in fig. 1, but receiving during 4 following weeks vitamins B and C. The foldings of the mucosa and the lumen as in fig. 2, but the epithelium is high as in fig. 1. Rat 245 g., uterus 248 mg.



FIG. 4.—Another ovariectomised rat treated as in fig. 3. Similar picture, but lumen like that in fig. 1. Rat 210 g., uterus 200 mg.





1. Squamous metaplasia in the uterine columnar epithelium (the usual effect of oestrogens—fig. 1) was apparently decreased. In the control rats receiving oestradiol alone (group I) considerable metaplasia of the uterine epithelium and uterine glands was recorded in 5 out of 8 rats, while in the 13 rats of groups II-IV metaplasia of slight degree was found in two rats only.

2. In 4 out of 9 rats (line 5 of the table) which received oestradiol+vitamin C (group III), or oestradiol+vitamins B and C together (group IV) foldings of the mucosa were observed (figs. 3 and 4) similar to those in the uterus of rats treated with oestradiol+progesterone (fig. 2). It is well established that a combination of oestradiol and progesterone causes typical progestational changes in both uterus and vagina. The progestational effect of vitamin C on the mucosal foldings did not, however, extend to the epithelial structure. The uterine epithelium of a pregnant rat or of an ovariectomised rat injected simultaneously with progesterone and oestrogens is low columnar with light vesicular nuclei (see figs. 77 and 82 in our previous paper, Korenchevsky and Hall, 1937), while that of a rat injected with oestrogens alone is very high columnar with darkish nuclei (*ibid.*, fig. 83). These changes are constant and typical.

In the present experiments the uterine epithelium of rats treated with oestradiol+vitamins was of oestrogenic structure (high columnar cells with dark nuclei), the mucosal foldings being the only typical progestational change.

There was no difference at all in the progestational changes of the uterus between groups V and VI. The "oestrogenic" metaplasia was completely prevented by progesterone in both groups (line 4 of the table), confirming our previous results (Korenchevsky and Hall, 1938) and those of other authors.

Taking into consideration the experiments of Biskind and Glick, and the fact that in our experiments a peculiar progestation-like change was obtained only in the rats which received vitamin C, this vitamin should be responsible for the change.

#### SUMMARY

1. On 31 ovariectomised rats the effects were investigated of the addition of extra amounts of crystalline vitamins B and C on the oestrogenic changes produced in the uterus and vagina by oestradiol alone, or on the progestational changes produced in these organs by the simultaneous administration of oestradiol and progesterone.

2. In rats injected with oestradiol, the addition of the extra vitamins did not affect the vagina, but ascorbic acid produced a peculiar combination of changes in the uterus—foldings of the mucosa typical of a progestational effect and very high columnar epithelium typical of the oestrogenic effect.

3. The vitamins did not alter in any way the progestational changes in the uterus and vagina produced by simultaneous administration of oestradiol and progesterone.

4. Metaplasia of columnar uterine epithelium into the stratified squamous type, usually produced by oestrogens, was completely prevented by the simultaneous administration of progesterone, and appeared to be considerably decreased by vitamins B and C.

We are indebted to the Medical Research Council and the Lister Institute for grants, to Messrs Ciba Ltd. for the hormones and to Messrs Roche Products Ltd. for a generous supply of vitamins.

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CHONDROSARCOMA OF A PHALANX WITH  
CUTANEOUS METASTASES

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(PLATES XVIII AND XIX)

There is a tradition that phalangeal chondroma is always benign. This view is succinctly expressed by Geschickter and Copeland (1936, pp. 91-92): "In the prognosis of these tumors, it is a striking paradox that the more cellular chondral lesions in the small bones associated with fetal cartilage and myxoma are uniformly benign; whereas the less cellular tumors of larger size occurring in the sternum and long bones, although composed of benign adult cartilage, must be looked upon clinically as potentially malignant". Ewing (1940) is somewhat less explicit and emphasises the necessity for careful consideration of the clinical as well as the histological features of the growth in formulating a prognosis.

A recent example of a cellular chondroma of a phalanx encountered in the Aberdeen Royal Infirmary has upset histological calculations by becoming widely disseminated in the skin of the face as well as in the lungs—a sufficiently disquieting occurrence to warrant a short descriptive note.

*Case report*

A man aged 66 had the middle finger of the left hand amputated on 5th March 1942 because of a tumour. Fig. 1 shows the X-ray appearances, and the radiologists's report stated "There is a large myxochondroma developing from the middle phalanx of the left middle finger. Its rather excessive non-calcified part suggests the possibility of malignancy developing as a secondary process in an old chondromyxoma". The histological appearances of the tumour are shown in figs. 2 and 3 and the following histological report was submitted. "The lesion is a chondroma. The tumour is very cellular, with some diversity in cell size and hyperchromatic nuclei but there is no sufficient justification for regarding the lesion as malignant. The probability is that the prognosis will be quite favourable".

On 16th July 1943 a biopsy from a lesion on the patient's right cheek was received and was diagnosed as a basal cell carcinoma in which there was some admixture of polygonal squamous cells. This lesion was cured by treatment with radium.

On 18th May 1944 the patient returned to the radium department because of nodules in the skin of the face and neck and to a lesser extent in the skin of the trunk. His appearance at this time is shown in fig. 4. He was breathless and X-ray examination of the lungs showed numerous rounded deposits of secondary tumour growth. Histological examination of a skin nodule revealed the appearances shown in fig. 5 and the nodule was reported to be a deposit of secondary chondrosarcoma with an apparently benign histological structure. The radium officer then made the diagnosis of multiple secondary deposits of chondrosarcoma from the tumour of the phalanx of the left middle finger removed in 1942 and mistakenly diagnosed as chondroma. Until the nature of the multiple skin nodules had been established by biopsy the patient had not mentioned the loss of his finger to the radium officer and the true nature of

## METASTASISING CHONDROSARCOMA OF PHALANX



FIG. 1—Chondrosarcoma of middle phalanx of middle finger showing extensive replacement of bone by growth, and a large extra osseous extension.

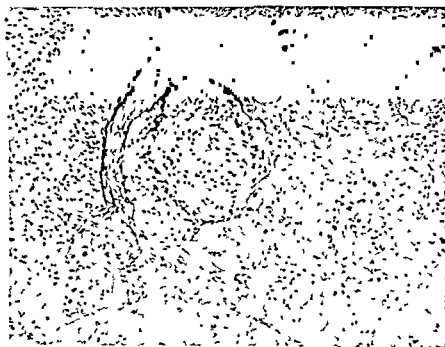


FIG. 2—Section of primary tumour, diagnosed as probably benign  $\times 35$



FIG. 3—Higher power view of primary tumour.  $\times 125$ .



METASTASISING CHONDROSARCOMA OF PHALANX



FIG. 4—Secondary deposits of chondrosarcoma in skin of face. Photograph taken 27½ years after amputation of the affected finger

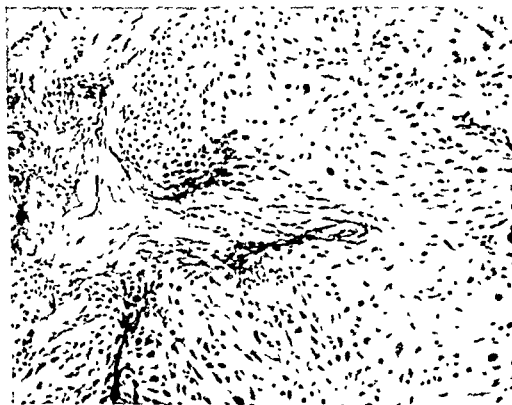


FIG. 5—Section of one of the cutaneous secondary deposits.  $\times 70$ .



the nodules had not been suspected. The stump of the amputated finger showed no obvious local recurrence.

This case is reported since it shows that Geschickter and Copeland's statement that cartilaginous tumours of the phalanges are uniformly benign requires modification.

### Summary

A case of chondrosarcoma of the middle phalanx of the left middle finger is recorded. Secondary deposits occurred in the skin and in the lungs. The histological structure of the primary tumour and of one of the cutaneous secondaries was apparently benign.

I am indebted to Mr J. F. Philip, radium officer, Dr J. E. Blewett, radiologist, and Mr S. G. Davidson, hon. surgeon, all of the Aberdeen Royal Infirmary, for permission to use their records of this case, and to Mr T. C. Dodds of the Pathology Department, Edinburgh, for the photomicrographs.

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547.9 (*Penicillin*) : 576.851.252 (*Staphylococcus*)

### THE VARIATION IN SENSITIVITY TO PENICILLIN OF STRAINS OF THE OXFORD STAPHYLOCOCCUS H

S. T. COWAN

*From a military laboratory*

The sensitivity of organisms to penicillin is expressed by some workers in terms of the sensitivity of the Oxford staphylococcus H (Garrod, 1944a); the validity of this comparison is based upon the assumption that the sensitivity of the reference strain remains constant. *Staphylococcus pyogenes* often produces variants differing from the parent strain in pigment or toxin production, and changes in the behaviour of an organism are most likely to occur when it is kept under different conditions. As the Oxford staphylococcus H has been widely distributed it may, in some laboratories, have developed characteristics not found in the parent strain. A comparison of seven strains of the Oxford staphylococcus H is described in this paper.

### *Cultural characters of the strains investigated*

Dr N. G. Heatley kindly sent me a dried culture of his organism and strains were also received from Professors L. P. Garrod and A. A. Miles, Dr E. T. C. Spooner, Lt.-Col. A. E. Francis, Major K. E. A. Hughes and the National Collection of Type Cultures.

All strains were coagulase-positive, fermented glucose, lactose and sucrose and acidified milk in 24 hours. Serologically all were type I, but the Spooner, Miles and N.C.T.C. strains were slightly less specific than the others and gave



feeble cross reactions with type III serum. The differences observed were that Miles's strain gave a gold pigmented colony, the others produced white colonies; mannitol was fermented in 24 hours by all strains except Miles's and Spooner's, both of which took 48 hours to ferment this sugar; and gelatin was liquefied in one day at 37° C. by all strains except those of Hughes and Miles (which took 5 days) and of Spooner (7 days).

### *Penicillin sensitivity*

Using doubling dilutions of penicillin in broth (Abraham *et al.*, 1941; Garrod and Heatley, 1944), the Hughes strain appeared to be slightly more sensitive than the others after 24 hours' incubation, but after 72 hours all strains grew in 0.05 unit per ml. and were inhibited by 0.1 unit per ml. The results obtained by this method were inconstant and irregularities such as those reported by Bigger (1944a) were not infrequent. Differences in sensitivity of the strains could be detected if decimal dilutions of broth cultures were plated on nutrient agar containing known concentrations of penicillin; the method, which is an adaptation of Miles's "surface viable count" (Aitken *et al.*, 1936; Kenny *et al.*, 1937), is described below.

### *Method of determining penicillin sensitivity*

One ml. of an appropriate dilution of penicillin was mixed with 19 ml. of agar at 50° C. and poured into a sterile petri dish. In these experiments final concentrations of 0.025 and 0.05 unit per ml. were used. Plates were dried in the incubator for about 30 minutes and the bottom of each dish marked into 20 rectangles ("squares") 1.5 × 1 cm. Tenfold dilutions (up to 10<sup>-4</sup>) of a 24-hour broth culture were made in peptone water. A platinum loop (1 mm. internal diameter) was dipped into the highest dilution, withdrawn vertically and used to inoculate the first square of a control plate without penicillin; a similar inoculum was spread over the first square of each plate in the order of increasing penicillin concentration. After flaming the loop the other dilutions of culture were plated in the same way, care being taken each time that the whole of the inoculum was spread on the medium. The plates were incubated at 37° C. and after 24 hours were examined in a good light. The control plate inoculated with a 10<sup>-4</sup> dilution usually had between 15 and 50 colonies. At the point where inhibition of growth occurred most of the colonies were small, but a few were larger and were believed to be a minority of more resistant organisms present in almost every strain of staphylococcus. One, two and occasionally three dilutions yielded a single colony; these were not "persisters" (Bigger, 1944b), as they had multiplied in the presence of penicillin. The end-point was taken as the largest inoculum from which not more than one colony developed. In titrations of one strain this end-point was shown to give more consistent results than complete suppression of growth or readings made after incubation for 48 hours.

### *Results*

Examples of titrations of the Oxford strains are shown in the table; the number of colonies which grew from the standard inoculum is stated except when they were too numerous to be counted, when + signs are used. The difference between some of the strains is clear. Repeated titrations do not always give exactly the same end-point but comparative tests show the same order of sensitivity. Spooner's strain was found to be unstable and readily threw off hypersensitive variants; the original sensitivity could be obtained regularly only in cultures made directly from the agar stab sent from Cambridge.

Some of the factors affecting the end point have been investigated. One of the greatest sources of variation is in the assay of the penicillin incorporated in the plates, the Oxford method of assay (Heatley, 1944) has been used and a mean taken of sixteen plates, by this procedure the experimental error is probably reduced to 10 per cent. The end point varied with the size of the inoculum, the least variation in multiple titrations of the same strain was obtained with a 1 mm loop. The variation in the inoculum delivered by the same 1 mm loop has been calculated from the counts of the  $10^{-6}$  dilution of one broth culture that was plated out 74 times, the mean count was 33.6, S.D. 6.83. Slight differences in penicillin concentration in the medium are believed to be responsible for the occasional variation between replicates on different plates. The results of several experiments showed that young cultures (4-8 hours) are more sensitive than older (20-24 hours).

TABLE

*Example titrations of the strains of Oxford staphylococcus*

Strain	Concentration of penicillin in the plates										Agar control
	0.025 unit per ml					0.05 unit per ml					
	Dilution of culture										
	1	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	1	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>
Spooner	+++	+++	+++	++	4	7	3	0	0	0	19
Heatley	+++	+++	+++	++	16	12	1	0	0	0	26
Francis	+++	+++	+++	++	37	16	0	0	0	0	35
Garrod	+++	+++	++	+	17	18	1	0	0	0	28
NCTC	+++	+++	++	+	36	0	0	0	0	0	13
Miles	+++	++	+	9	0	0	0	0	0	0	64
Spooner (variant)	++	+	23	0	0	0	0	0	0	0	24
Hughes	++	+	8	0	0	0	0	0	0	0	24

Numbers indicate the colonies which grew from the standard inoculum

+++ = confluent growth

++ = semiconfluent growth

+ = colonies too numerous to be counted

### Discussion

The wide distribution of Heatley's strain of *Staphylococcus pyogenes* has afforded a unique opportunity to study the changes induced by different environments. Strains of this organism from seven laboratories have similar cultural and serological characters except for some slight change in the proteolytic activity of certain strains. Sensitivity to penicillin differs among the strains and three of them (Miles's, Hughes's and a variant from Spooner's) form a hypersensitive group. Daily subculture in broth had been used by Hughes for the maintenance of his strain and it was found to be nearly twice as sensitive as Heatley's (the dried culture was the second subculture from the original). Garrod's strain, which had retained the sensitivity of the original culture, had been subcultured once a month and plating with colony selection had been avoided. If one strain of staphylococcus is to be used for reference purposes it is desirable that the conditions for its maintenance should be defined and as Garrod's strain has retained the sensitivity of the original organism it is suggested that his procedure be adopted.

The variation in sensitivity shown in this paper affects comparisons of unknown organisms with the "Oxford staphylococcus" but it does not influence the results of assays of penicillin by the Oxford method, as dilutions of standard penicillin and the unknown are titrated on the same plates.

Abraham *et al.* (p. 182) state that "The action of penicillin on streptococci and staphylococci, unlike that of the sulphonamides, is only influenced to a minor extent by the number of bacteria to be inhibited". This statement appears to have been accepted (Garrod, 1944b), but Spink *et al.* (1944) make a reservation excluding resistant staphylococci. Rantz and Kirby (1944) found that the size of the inoculum was important in showing differences between strains and advocate a large inoculum. The results obtained with the technique described in this paper suggest that with staphylococci there is a concentration of penicillin critical for each strain and that at this level the size of the bacterial population becomes material.

### Summary

Seven strains of the Oxford staphylococcus H were compared and three were found to be more sensitive to penicillin than the dried culture received from Oxford. These differences could not be detected by the usual methods but were shown by inoculating falling dilutions of culture on plates containing varying concentrations of penicillin.

I wish to thank Major-General L. T. Poole for the opportunity and facilities for carrying out this work and the bacteriologists named in the text for their kindness in sending me their strains of the Oxford staphylococcus H.

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578.67

## POST FIXATION OF TISSUES

E H LEACH

*From the University Department of Physiology, Oxford*

During the last two decades of the nineteenth century several successful methods were worked out for affixing paraffin sections to glass slides. Previous to this, sections had been stained and mounted in the same manner as frozen sections, a process requiring much care and patience. As it became possible to cut thinner sections, this method of mounting paraffin sections became more than ever desirable. The success of the methods have been judged, not on the absence of visible damage caused to the tissues, but on the perfection of adherence during the staining processes.

Of the many methods which have been suggested the following are still in use

Gaule (1881, p. 156) flattened sections out on alcohol. This method is used for flattening out sections to demonstrate glycogen and was found by Leach (1938) to be superior to later methods for demonstrating muco protein.

Mayer (1885) smeared a concentrated solution of egg albumin and glycerine on the slide. It is usual now to add distilled water.

Duval (1891) used a dilute solution of glycerine and egg albumin.

Gulland (1891 '92) found that water alone, without any albumin, could be used. This method is often used in this laboratory when large numbers of sections have to be mounted. The sections are placed on a bowl of warm distilled water and drawn on to clean slides.

Mann (1892 '93) used slides which had been albuminised and then allowed to dry. The sections are flattened out on a drop of distilled water on the slide.

No mention is found in the literature of those times of any attempt to see whether these flattening out processes caused any damage when the sections were compared with those treated individually in small vessels. It is not worthy that some careful histologists several years later were still not flattening out paraffin sections before staining. This is particularly noticeable in work on muco protein, e.g. that of Maximow (1906). Mann (1902) seems to have been aware of the possibility of damage and has stigmatised such treatment as "unconsciously undoing good fixation." But this cannot have seemed to him to cause important damage, for he advises flattening out of sections on water placed on albuminised slides.

Hardy and Westbrook (1895), in a careful study of leucocytes in tissues, are very positive about such damage. They found that not only must water be prevented from coming in contact with the sections at any stage but that even moist air might cause the muco protein granules of mast cells in the villi of the intestine to swell up and disappear. The alcoholic staining solution used might not contain more than 15-20 per cent of water. This confirmed similar results of List (1885). Weski (1901) found that like precautions must be taken in the staining of eleidin. Such observations are explicable on the assumption that the substances involved cannot be rendered insoluble despite fixation dehydration etc. It is obvious that if watery solutions are found to cause damage in the staining process, sections may not be flattened out on water (Hardy and Westbrook, Weski).

But it has also been noted that if water is avoided in the flattening out stage, aqueous solutions may still be used in staining. Such a phenomenon is so surprising, and at first sight so unlikely, that it is understandable how it has been overlooked. Bizzozero (1892) found that, in studying muco-protein,

sections must not be flattened on water but could later be treated with water. This was confirmed by Leach, who flattened sections on 96 per cent. alcohol. Carleton and Leach (1939) found that, if sections were flattened on 50 per cent. diacetin, greatly improved cytological and pathological preparations could be obtained with staining techniques involving the use of aqueous solutions. Unfortunately there is a difficult technique to use and cannot be advised for routine work. The assumption then made was that, following the removal of fatty substances from tissues, unfixed proteins remain and can be fixed by flattening the section on a protein precipitant such as alcohol or diacetin. This would explain why treatment with water immediately after fixation or during staining was possible, while such treatment after clearing in xylol but before treatment of sections with alcohol was not permissible. This view has been accepted by Baker (1941), who suggests that it explains why he had got such good cytological preparations when the cut face of a paraffin block was treated with protein precipitants.

It has been found that the method here described gives results which, although not quite as good as those seen after flattening sections on diacetin, nevertheless show a great improvement on those obtained by the more usual methods. It is easy to use and can readily be applied to routine work.

#### *Method*

1. Fix by any desired method. Wash in water if customary.
2. Dehydrate with alcohol.
3. Clear in xylol for 3-12 hours (change once).
4. Treat with absolute alcohol for 6-24 hours (change once). This stage has been termed "post-fixation".
5. Clear by any desired method.
6. Embed in paraffin; soften block in water if necessary before cutting sections.
7. Flatten out sections as usual on water or egg albumin solution.

Stages 3 and 4 are the only departure from normal technique, which may otherwise be retained in whatever form is customary.

#### *Results*

The method described has been in use for three years. Until a technique was shown to be improved by this method, control material, which remained in alcohol whilst the other was being post-fixed, was always embedded in the usual way. During this time a large variety of tissues, fixatives and staining methods has been used. The tissues cut at least as well on the microtome and the sections flatten at least as well on water. The following improvements have been seen in sections prepared by this method as compared with those prepared by the normal method, strict care being taken to use comparable pieces of tissue and to stain control material on the same slide.

1. General nuclear and cytoplasmic fixation is improved. Granules stain more sharply. Cell boundaries and intracellular membranes are particularly well preserved. These improvements are most noticeable when fixation is poor.

2. Post-mortem changes are much reduced.

3. Muco-proteins are better preserved.

4. Mitochondria stain more intensely and more sharply with iron hæmatoxylin after formol fixation and post-chroming. Their form and position are more like those described in the most carefully prepared preparations.

5. Phosphatase demonstrated by the method of Gomori (1941) is more sharply localised and general fixation is improved.

6. Vitamin C preparations made by the method of Barnett and Bourne (1940-41) show much improved general fixation.

When sections prepared by this method are flattened out on diacetin still

further improvement in cytological fixation is observed. The Péterfi (1921) celloidin double embedding method, which even without post fixation gives improved cytological preservation, gives even better results with it. Similar improvement is seen when material for celloidin embedding is post fixed.

### Discussion

The theoretical basis for this method is probably the same as that advanced to explain the success of the diacetin technique. The treatment of tissues with xylol removes fatty substances which have chemically or physically protected some proteins from the initial fixation. Subsequent treatment with alcohol can complete the fixation and prevent alteration or solution of substances when the sections are flattened on water. As would be expected from this hypothesis, better preservation of the shape of cells and their constituents is observed and greater staining affinity is obtained. This is particularly true of lipo protein structures such as cell membranes and mitochondria. Because some special cytological techniques may depend for their success on artefact changes during the normal process of preparation, it is advisable to embed control material by the usual methods until it has been shown that this new method gives, as it has done so far, results which are as good or better. But such a precaution can certainly be dispensed with when common histological and pathological fixation and staining methods are used.

It seems probable that the success of the flattening out method of Gulland depends on the solution of unfixed proteins from the section. These will then replace the albumin that is normally used to cause adherence of the section to the slide. It would be unwise to use this method on post fixed material in which coagulation of these proteins might have been caused prior to embedding.

That further improvement can be obtained by flattening on diacetin the sections produced by this method is readily understandable. When sections are flattened on water the tissues swell. Subsequent contraction on drying is limited by adherence to the slide. Distortion is inevitable. When flattened on diacetin the swelling and contraction are usually less and the contraction can take place freely because the tissues do not adhere to the glass.

The success of the Péterfi double embedding method, whether applied to normal or post fixed tissues, probably resides in the coherence given to the tissues by the celloidin. The swelling and contraction of the tissues in the sections when flattened out on water and then dried will be limited and made more even.

It is likely that "good" fixatives such as Carnoy rely for some of their success on their ability to fix those proteins which are protected from bad fixatives such as 80 per cent alcohol by the presence of fatty substances. This would account for the smaller success of this new method after good fixation than after bad, which has sometimes to be used for special purposes.

The marked diminution in post mortem degeneration when material is post fixed is of interest. It indicates that much of the morphological damage which has been considered to have occurred prior to fixation has not in fact taken place by that time. Instead it must now be thought that prior to fixation a delicate change occurs in the nature and distribution of the lipid substances. This is revealed by the subsequent lability of the proteins when sections are flattened out on water. Post fixation prevents these morphological changes in the distribution of the proteins.

### Summary

A simple and reliable method is described whereby improved fixation of tissues for paraffin embedding may be obtained by post fixing them in alcohol after treatment with xylol.

The method is particularly applicable when for special purposes fixation has to be done in a bad fixative or when post-mortem changes are likely to have occurred.

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## BOOKS RECEIVED

### Manual of human protozoa

By RICHARD R. KUDO. 1944. Springfield, Ill., and Baltimore, Md.: Charles C. Thomas. (Distributors in the British Isles, Baillière, Tindall & Cox.) Pp. ix and 125; 29 text figs. Price 11s.

In 1931 Dr Kudo published his now well known *Handbook of protozoology*, a work which received high praise from the medical scientific press as a book of general reference for those interested in human and veterinary protozoology. In 1939 there appeared an enlarged and completely rewritten edition, consisting of some 700 pages of close print and nearly 300 illustrations. The revised edition contained many additional references to the more important discoveries in medical and veterinary protozoology, but, as was inevitable in a work dealing with so vast a subject, the author was again compelled by the exigencies of space to devote but little attention to the specialised needs of the medical profession. He has now attempted to meet this need by the publication of the present short manual of human protozoology.

Dr Kudo has produced a book, which, as might be expected from a scientist of his distinction, is accurate, concise and informative, both in letterpress and in illustrations, and it can safely be recommended as a reliable elementary guide to the student of human protozoology, so long as it is used in conjunction with some other work which deals more fully with the development and habits of the parasites studied. The reason for this advice is perhaps best explained by a quotation from the author's preface. "There are now available several excellent treatises on parasitic protozoa of man which deal comprehensively with the morphological and developmental details, incidence of infections, geographical distribution, pathogenic changes brought about in the human body, diagnosis, treatment, etc. As no one of these meets the need of the class, the present manual has been prepared. It contains only the essential information in order to serve as a practical guide or companion book in detecting and identifying the human protozoa". To be able to detect and identify the pathogenic protozoa is obviously essential, but can the student appreciate the methods employed to detect and identify the parasite, without knowing about its "developmental details"? If he knows nothing of its geographical distribution can he appreciate the fallacy of searching for trypanosome infection in a man who has only voyaged to India? If he has no information concerning the incidence of the parasite, can he realise the extreme caution which is necessary before diagnosing coccidiosis in the human host? The reviewer hastens to add that Dr Kudo's manual does supply this information in the case of some of the parasites described, but the general tendency of the book is to omit the biological aspect and to describe the parasites as if they were inanimate objects, instead of animate creatures ever changing in form and habits during their curious and complicated life-cycles. It is the reviewer's contention that knowledge of these habits and changes is an essential preliminary to the detection and identification of the parasites. Such information is absent from, or insufficiently given in, Dr Kudo's Manual, while a surprising amount of space is devoted to subjects which most medical protozoologists would regard as relatively unimportant. Thus, almost three pages are devoted to an account (which includes signs and symptoms observed in a single case) of *Isospora hominis*, and five pages to such comparatively rare organisms as *Sarcocystis lindemanni*, *Retortamonas intestinalis* and *Tricercomonas intestinalis*. Three pages of



text are occupied by accounts of the mouth-inhabiting protozoa *Entamoeba gingivalis* and *Trichomonas elongata*, and five pages to coprozoic protozoa which "only occur in faeces after it has been voided". In a work intended for elementary instruction it is surely unnecessary to print a table giving the strength of various chemicals and drugs which are lethal to the cysts of *E. histolytica* at different temperatures, and still less necessary to record the temperatures which are sufficient to destroy *E. gingivalis* at various intervals of time. Such prodigality of unessential information is out of proportion in a book which devotes only five pages to the life-history and recognition of the predominantly important species *Entamoeba histolytica* and less than four pages to the genus *Leishmania*.

The book is beautifully produced and printed, and the illustrations, although some have suffered by their reduction in size, are excellent.

R. M. GORDON.

#### Statistical methods for research workers

By R. A. FISHER. 9th ed. 1944. Edinburgh: Oliver and Boyd. Pp. xv and 350; 12 text figs. 16s.

The fact that this book has now reached its ninth edition is the best evidence that it succeeds in serving the purpose for which it was written. The period since 1925, when it first appeared, has seen a change in the attitude of the average worker in the pathological sciences towards statistical methods. In those days the mathematical treatment of data was regarded by many with suspicion, and the statement that "you can prove anything by statistics" was frequently heard. Much of this was due, as Professor Fisher says in his preface, to the fact that the traditional methods were wholly unsuited to the needs of practical research. In successive editions of his book new methods have been formulated, and it would not be too much to say that the present more rational attitude to statistical methods has been to a large extent a result of its publication.

In the ninth edition the general plan of the work follows previous lines, and there is a new section on the test of homogeneity of evidence used in estimation. There is one small point in which it appears to the reviewer that the lay-out could be clarified; a slight separation between the literary punctuation and the equations and other mathematical expressions which it follows would sometimes prevent possible confusion in the mind of the non-mathematical reader. When, for instance, a comma follows the symbol  $n'$  as closely as it does on p. 110, he may turn back to find out what " $n$ " means.

The decimalised system of numbering sections, tables and examples allows new material to be inserted without altering the numbers of existing sections, etc., so that references to them, but not to pages, are valid in any edition. It is important that authors should remember this in quoting from this book, especially in these days when any given edition may be difficult to obtain.

#### Sternal puncture

By A. PINEX and J. L. HAMILTON-PATERSON. 2nd ed. 1943. London: William Heinemann (Medical Books) Ltd. Pp. xi and 69; 13 plates (12 in colour) and 2 text figs. 15s.

The second edition of this monograph, appearing within two years of the initial publication, is a distinct improvement on its predecessor. Without increasing the size of the volume, several new sections have been added, including a more adequate discussion of the anæmias, a description of the reticuloses affecting the bone marrow and additional technical

procedures of use to the hæmatologist. An attempt has been made to remedy a great defect in the earlier edition by the inclusion of twelve colour plates. While the new colour frontispiece is a distinct improvement, many of the other illustrations are of indifferent quality, particularly that of the megaloblastic and normoblastic derivatives. Although the authors attempt to differentiate the myeloblast from the lymphoblast on the morphology of individual cells, their illustrations of acute myeloid and acute lymphatic leukaemia are not convincing except for the inclusion of a typical myelocyte in their plate of the former. The bibliography has been revised to include some of the more useful recent contributions to the subject.

While a volume of this size cannot cover the subject as adequately as our present knowledge of hæmatology would seem to warrant, the new edition contains much of use to the laboratory worker.

#### A pathology of the eye

By EUGENE WOLFF. 2nd ed. 1944. London: H. K. Lewis. Pp. vii and 285. 216 figs., 5 in colour. £2, 2s.

Morbid histology is given greater attention in this second edition, and the text, with evident benefit, has been largely rewritten; 88 new figures are added. The text, however, is still marred in places by looseness of expression and the retention of terms and opinions which, if not obsolete, are out-dated as judged by modern standards. "Cataloguing", exemplified by the section on colobomata, is still prevalent and some subjects of interest and importance, for example, uveoparotid fever, are dismissed cursorily, in this instance to leave a blank half-page below. The chapter entitled "Some points in general pathology" is entirely superfluous, and a poor compliment to potential readers. The implied emphasis, in text and legends, on the diagnostic importance of the giant cell in tuberculosis is overdone. At least as important is the recognition of follicular structure, even in the absence of giant cells.

Many of the new figures are of inferior quality when compared with the high standard set by their predecessors, and some are wholly unworthy. The two plates in colour might have been put to much greater use. One is allotted to the normal anatomy of the optic nerve head, the other to *siderosis bulbi*. A comprehensive bibliography, especially of references since 1908, would have commanded, of itself, the interest of a wide field. The present bibliography, despite its range, is unsatisfactory. The important subject of intra-ocular new growths is represented by less than thirty references, while eight are allotted to the unimportant subject of *siderosis bulbi*.

For some time there has been a distinct need for either a modern edition of Parsons' *opus* or its equivalent. The present volume in some degree fills the gap, but it is disappointing. Supported apparently by ample funds, a superb opportunity has not been fully seized.

The production of the book is beyond criticism and the text is almost free from typographical errors. The post-graduate student and the general pathologist, occasionally required to examine and report upon eye material, will find this book helpful, but those who specialise in ophthalmic pathology are likely to be dissatisfied.

#### Clinical laboratory methods and diagnosis

By R. B. H. GRADWOLD. 1943. London: Henry Kimpton. Two vols, pp. xxiii and 2130; 726 text figs. and 57 plates in colour. £5.

The second edition of this monumental work appeared in 1938, three years after the publication of the first. Now the third edition has appeared,

this time in two still weighty volumes instead of the previous rather clumsy single one. The number of pages has been increased by some 700, including the printing of the index in duplicate, one for each volume; more text illustrations and colour plates have been included and the price has been doubled.

The second edition has been in use in the reviewer's laboratory for several years and it is probably true to say that on no single occasion has it been consulted in vain. It is a most valuable book of reference and the addition of a new discussion on liver function, more details of blood grouping (including the Rh factor) and a section on vitamin assay and identification will enhance its value. Especially useful is the description of the bone marrow in health and disease.

The chief criticism of this book lies in its inequality of emphasis. For example, on pp. 1079 and 1149 illustrations and descriptions of methods of drawing capillary pipettes and pouring agar plates respectively are given. The illustrations could easily have been dispensed with. But, although the omission was pointed out in this *Journal* in a previous review, there is still no reference to the use of the Price-Jones curve in the measurement of red cells, nor is a really accurate method for the estimation of fragility of red cells given.

The advisability of including all aspects of clinical laboratory methods and diagnosis in one book is a matter of opinion. For workers in isolated laboratories such a mine of information as this obviously fulfils a real need.

#### Stitt's Diagnosis, prevention and treatment of tropical diseases

By RICHARD P. STRONG. 7th ed. 1944. Philadelphia: The Blakiston Co. Vol. I, pp. xvii, 871 (plus interpolated pages) and xl; 198 text figs. and 4 plates (2 in colour); vol. II, pp. vii, 874 (plus interpolated pages) and xl; 201 text figs. \$21.

This valuable and well known work appears once more in two handsomely produced volumes as the seventh edition in the series and the second under the editorship of R. P. Strong. Its content is that of its predecessor (noticed in this *Journal*, 1942, liv, 405), although its bulk is a trifle less owing to the use of thinner paper. The text has undergone only minor modifications directed towards bringing it up to date where advances in knowledge, often a result of war experience, have indicated the need for elaboration or change. Examples of this are found in the sections on the treatment of bacillary dysentery, on the vaccine prophylaxis available against epidemic louse-borne typhus, on the newer insect repellents used in malaria prophylaxis, and on the drug treatment of kala-azar and of the trypanosomiasis and spirochaetoses. Scrub typhus receives rather more attention in view of its manifest importance in the war in the Pacific area, and a new clinical condition, Bullis fever, of rickettsial origin and conveyed by the tick *Amblyomma americanum*, is briefly described. There remain a few items which are in need of revision; for instance the treatment of scabies, where neither benzyl benzoate nor the newer drugs are even mentioned.

Any increases in printed matter have been offset by the greater use of small type or by the insertion of odd pages such as 576a and 576b: apart from this, page for page the present edition exactly corresponds with its immediate predecessor, even to misprints, few as these are. The volumes continue to fulfil well the increasingly world-wide general demand for a reliable and lucid text-book in the English language on those conditions commonly, though often erroneously, referred to as tropical diseases.

# The Journal of Pathology and Bacteriology

Vol. LVII, No. 2

616.15—097.5 (blood group factors A, B and Rh)

## ISO-IMMUNISATION TO THE BLOOD GROUP FACTORS A, B AND RH \*

K. E. BOORMAN, B. E. DODD AND P. L. MOLLISON

*From the S.W. London Blood Supply Depot*

ISO-IMMUNISATION is the process by which immune antibodies are produced in one individual in response to the injection of blood, from another individual of the same species, containing antigens which the first individual lacks.

The first experiments on this subject were made on animals (*e.g.* Ehrlich and Morgenroth, 1900) Dienst (1905) suggested that there might be iso-immunisation within the human species to the blood group factors A and B. He and later Levine (1943) detected immune anti A and anti B iso agglutinins in maternal sera following pregnancy. Jonsson (1936) showed an increase in anti-A and anti-B iso haemolysins in recently delivered women. Immune anti A and anti B iso agglutinins have also been demonstrated in the sera of persons who had received an incompatible blood transfusion (Ro, 1937; Wiener *et al.*, 1941, Lauer, 1941; Mollison and Young, 1941; Drummond, 1944). Unidentified atypical antibodies were found by Landsteiner *et al.* (1927-28), Neter (1936) and Zacho (1936) in individuals who had received repeated transfusions of homologous blood. The finding of immune anti-Rh iso-agglutinins after homologous blood transfusion or pregnancy has been reported by many workers, *e.g.* Wiener and Peters (1940), Levine *et al.* (1941), Boorman *et al.* (1942), Race, Taylor, Cappell and McFarlane (1943).

The object of the present investigation was to study in greater detail immune responses to the blood group factors A, B and Rh in the human subject following incompatible blood transfusion and

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\* A report to the Medical Research Council

Other aspects of some of the cases here reported have been published previously (Boorman, Dodd and Mollison, 1942, 1944, Mollison, 1943).

pregnancy. As it is now established that hæmolytic disease of the foetus is usually due to the presence of immune anti-Rh iso-agglutinins in the mother's serum, the clinical condition of the infant was noted in every case in which such immune iso-agglutinins were produced following pregnancy. For our purpose the clinical condition was assessed under four headings: (a) hæmolytic disease of the foetus, (b) stillbirth (including miscarriages), (c) physiological jaundice, (d) normal. Criteria for the diagnosis of conditions (a) and (c) are given by Boorman *et al.*, 1944.

As far as possible, the titre of the iso-agglutinin was determined both before and after stimulation by the homologous agglutigen and was estimated at close intervals throughout the response.

### TECHNIQUE

*Taking of samples.* Samples were withdrawn from the patient's vein using a dry sterile syringe. They were allowed to clot and the sera free from red cells were used for the titrations. A 1 per cent. suspension of red cells from the clot was used for grouping and Rh typing. If it was not possible to titrate the serum immediately it was stored frozen solid at  $-15^{\circ}\text{C}$ .

*Grouping of erythrocytes.* The method of ABO grouping was the tube technique described by Taylor *et al.* (1942) and of the Rh typing that described by us (Boorman *et al.*, 1942).

### *Titration of iso-agglutinins*

*Anti-A and anti-B.* The method of performing and reading the titrations is a slight modification of that described by Taylor and Ikin (1939). The titrations were carried out in  $2 \times \frac{1}{2}$  in. round-bottomed precipitin tubes, 0.85 per cent. solution of sodium chloride being used as diluent. The standard red cells were obtained from the same donors throughout and were always less than 24 hours old when used; a 2 per cent. suspension (in terms of whole blood) was made in 3.8 per cent. sodium citrate solution. With a Pasteur pipette graduated to deliver a constant unit volume (approximately 0.04 c.c.) twofold dilutions of serum were made in a row of tubes. To each of these serial dilutions one volume of the appropriate red cell suspension was added and the tubes shaken. The titrations were made in duplicate and were allowed to stand for 2 hours at room temperature. The cell deposit was then agitated by tapping sharply and the degree of agglutination read. No reaction was scored as negative until it had been examined microscopically. An even distribution of clumps of 3 or more cells was accepted as evidence of agglutination. The titre of the serum was expressed as the reciprocal of the greatest dilution causing agglutination.

The experimental error of this technique in the hands of an experienced worker very rarely exceeds one dilution, in spite of the fact that the unit volume employed is small. The titration values are higher than those obtained when the serial dilutions are made with standard graduated pipettes using a separate pipette for each dilution, because inevitably there is a slight carry-over from tube to tube when the same pipette is used for the whole titration. For the purpose of this investigation this factor was unimportant, because when titrating iso-agglutinins to estimate an immune response the object is merely to obtain comparative values.

*Anti-Rh.* The technique was essentially the same as that adopted for anti-A and anti-B. Each serum was titrated against erythrocytes from two Rh-positive individuals, one belonging to the subgroup Rh<sub>1</sub> (genotype Rh<sub>1</sub>Rh<sub>1</sub> :

see Race and Taylor, 1943) and the other to subgroup Rh<sub>2</sub> (see Race, Taylor, Boorman and Dodd, 1943). Control tubes containing one volume of Rh-negative cell suspension with one volume of serum were included in each test. As the anti-Rh agglutinins are more active at 37° C. than at room temperature (see table II, p. 167) the tests were kept at 37° C. for at least 2 hours before being read. Owing to the ease with which Rh agglutination can be broken down by rough handling, the contents of each tube were treated with great care and gentleness. The tubes were never sharply tapped but some of the sediment was gently removed by means of a Pasteur pipette and placed on a microscope slide. Clumps of 3 or more cells were again accepted as evidence of agglutination, and the titre of the serum was taken as the reciprocal of the highest dilution at which these were seen.

## IMMUNE RESPONSES TO THE AGGLUTINOGENS A AND B

### *Incompatible blood transfusion*

During the past 3 years we have had the opportunity of studying the iso-agglutinin changes in 5 patients to whom varying amounts of blood of an incompatible ABO group were inadvertently transfused (table I). All the recipients were of group O, 3 received group A

TABLE I

*Responses following transfusion of blood of incompatible ABO group*

Case no.	Blood group of recipient	Group of blood given	Approximate amount of blood given (c.c.)	Recipient's iso-agglutinins	Titre of iso-agglutinins in recipient's serum												
					Days after transfusion												
					1	2	3	5	7	8	10	11	14	18	20	30	35
1	O	AB	10	anti-A	...	...	...	128	...	1000	...	...	...	...	...	...	...
				anti-B	...	...	...	32	...	512	...	...	...	...	...	...	...
2	O	A	45	anti-A	512	...	2000	...	...	8000	...	32000	4000	...	256	...	256
				anti-B	128	...	128	...	...	32	...	128	128	...	64	...	128
3	O	A	1000	anti-A	...	...	...	...	...	...	256000	...	32000	8000	...	...	...
				anti-B	...	...	...	...	...	...	512	...	2000	512	...	...	...
4	O	B	600	anti-A	256	256	256	256	...	...	...	...	...	...	...	...	...
				anti-B	8	8	64	4000	...	...	...	...	...	...	...	...	...
5	O	A	50	anti-A	128	...	1000	...	1000	...	...	...	8000	...	...	2000	...
				anti-B	128	...	256	...	256	...	...	...	256	...	...	128	...

blood, 1 group B and 1 group AB. Both anti-A and anti-B iso-agglutinins were titrated in each case. The iso-agglutinin corresponding to the group of the blood given was found to increase in titre, a peak value being attained between the tenth and twentieth days after transfusion, following which the titre returned slowly towards its normal level. There was no change exceeding the normal variation in the titre of the other agglutinin. Thus the increase observed was due to a specific immune response. Case 4 is of interest because the specimens obtained soon after the transfusion had a very low anti-B titre (8) compared with the anti-A titre (256).

It seems probable that in this case some of the naturally occurring anti-B iso-agglutinin had been neutralised by the transfused group B blood. By the fifth day after transfusion, however, the immune response had increased the anti-B titre to 4000, the anti-A titre remaining constant. Unfortunately at this stage the patient died and it was not possible to estimate her normal anti-B titre.

As has been pointed out by previous authors (Wiener *et al.*, 1941; Mollison, 1943), in cases where an incompatible blood transfusion is suspected and no information with regard to the donor's blood group can be obtained, it may be helpful to titrate the iso-agglutinins in the recipient's serum. The most suitable time to do this is 5-10 days after the transfusion, as the increase in titre is likely to occur during this time. An unusually high titre of agglutinins for A or B or for both will indicate the probable group of the transfused blood.

The behaviour of the anti-A and anti-B iso-agglutinins in several recipients who received homologous blood was also studied. There was no change in the titre of either agglutinin greater than the normal variation (see Aubert *et al.*, 1942, fig. 1).

### *Pregnancy*

In the present investigation 3 groups were studied: (1) cases in which the maternal serum contained anti-A agglutinins and the infant's erythrocytes the A agglutinin (*i.e.* mothers of groups O and B with infants of groups A and AB); (2) cases in which the maternal serum contained anti-B agglutinins and the infant's erythrocytes the B agglutinin (*i.e.* mothers of groups O and A with infants of groups B and AB); (3) a control series in which there was no maternal agglutinin capable of acting on the infant's erythrocytes. Samples of maternal blood were taken as soon as possible after delivery (usually within 24 hours) and then at intervals of a few days for about a month.

**Group 1.** Of the 29 cases in this group 25 showed a marked increase in the anti-A titre following delivery. In the remaining 4 no immune response was detected. Fig. 1 summarises the results in the 25 cases in which there was an immune response. The normal anti-A titre in these cases varied between 32 and 1000. The "degree of immune response" (fig. 1a) indicates the number of times the peak titre recorded was greater than the patient's normal (*e.g.* the degree of response was 8, if the normal titre was 16 and the peak attained 128). The titre at delivery was taken to be the patient's normal, although there may in some cases have been an increase in titre during pregnancy. Such increases during pregnancy are usually quite small (unpublished observations). Fig. 1b shows that the peak titre is most often attained between the tenth and twentieth day after delivery, although it may be rather earlier or later in individual cases. The actual peak titres recorded varied between 512 and  $>256,000$  (fig. 1c).

**Group 2.** Among the 15 cases in which the mother belonged to group O or A and the infant to group B or AB, there were 4 in which no immune response could be detected. The results in the other 11 cases were similar to those in group 1. The degree of the immune

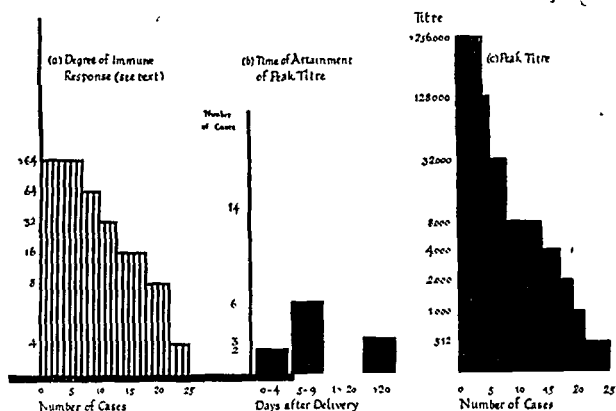


FIG. 1.—Summary of 25 cases in which an immune anti-A iso agglutinin was produced in the maternal serum in response to stimulation by a group A or AB foetus.

response varied between 4 and 64 and the peak titres between 512 and >256,000. The peak titre was again reached between the tenth and twentieth day after delivery in the majority of cases (7 out of 11).

**Group 3.** Thirteen cases were studied in which there was no incompatibility between the mother's agglutinins and the infant's erythrocytes. The maternal agglutinins were titrated at intervals, as in groups 1 and 2. The changes in titre in this series in no case exceeded the normal limits of variation.

*Graphic representation of immune responses.* If the titre of the iso-agglutinins in the patient's serum is plotted at various intervals, a curve similar in shape is attained for all the immune responses described above, whether the stimulus is an incompatible blood transfusion or a pregnancy. A typical example is shown in fig. 2.

#### IMMUNE RESPONSES TO THE AGGLUTINOGEN RH

Wiener and Peters, while investigating intra-group hæmolytic transfusion reactions, found immune anti-Rh agglutinins in some Rh-negative recipients transfused with Rh-positive blood. Levine *et al.* demonstrated that anti-Rh agglutinins could also be produced in an Rh-negative mother in response to stimulation by an Rh-positive



foetus. In these cases, however, details of the immune responses were not given. In our series we have studied the immune response of Rh-negative individuals (a) following transfusion with Rh-positive

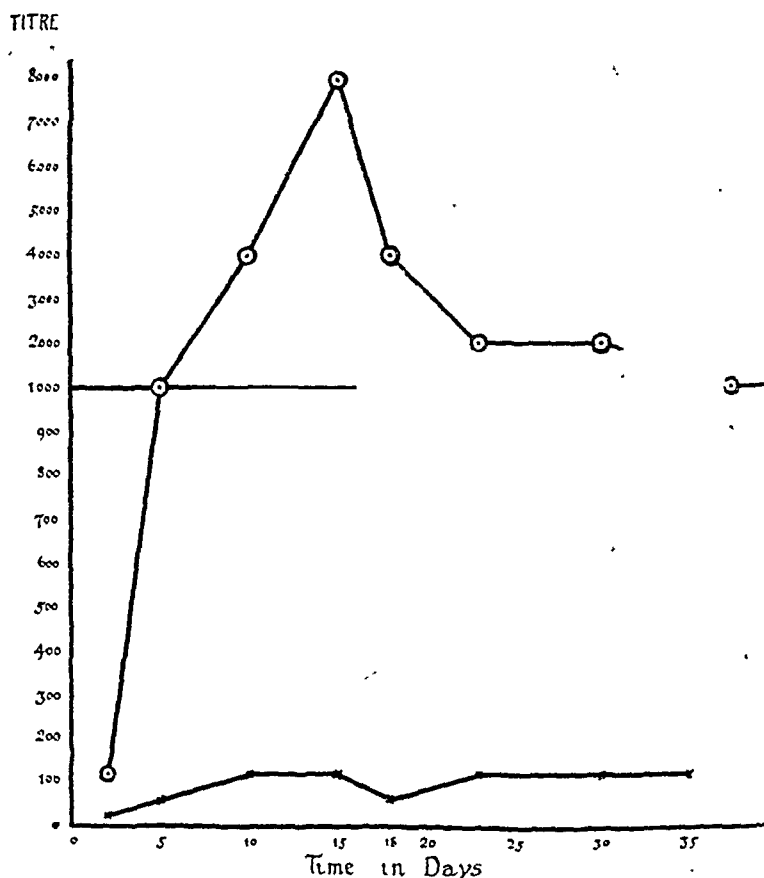


FIG. 2.—Typical immune response (anti-A).

Key to figs. 2, 3, and 5.

—○— Titre of anti-A iso-agglutinin.

—×— Titre of anti-B iso-agglutinin.

— — — Titre of anti-Rh iso-agglutinin.

blood, (b) after the delivery of an Rh-positive child and (c) following transfusion with an Rh-positive blood immediately prior to or just after delivery of an Rh-positive child.

#### *Rh-positive blood transfusion*

Rh-negative individuals seldom produce immune anti-Rh iso-agglutinins after only a few transfusions of Rh-positive blood. However, five such cases have been encountered and the immune response

in one of these is shown in fig. 3. By the fifth day after transfusion only a very small percentage of the transfused erythrocytes were left in the recipient's circulation and anti-Rh agglutinins were present in the serum (titre 8). By the seventh day, the donor cells were completely eliminated and the titre of the anti-Rh agglutinin had risen to 32. Eighteen months previously this patient had received two transfusions comprising blood from five donors, two of whom were subsequently tested and found to be Rh-positive. We were not able to study the other cases in such detail, but in each of them there was an unduly rapid elimination of the transfused erythrocytes followed by the appearance of anti-Rh iso-agglutinins in the recipient's

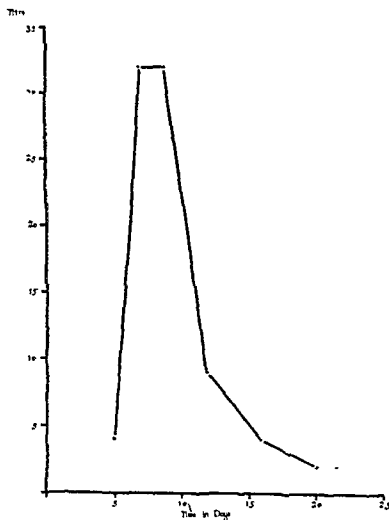


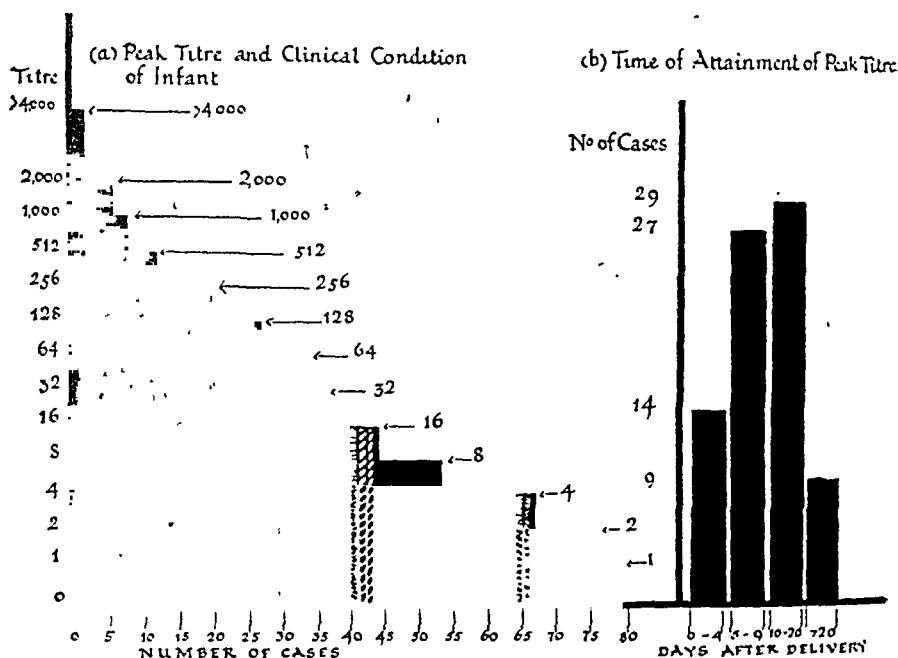
FIG. 3.—Anti-Rh immune response in an Rh-negative individual following transfusion of Rh-positive blood.

serum. In one case (see Dacie and Mollison, 1943), they were produced in response to a single transfusion, the transfused erythrocytes in this case surviving for about 2 months. In each case the peak titre was attained after the complete elimination of the transfused erythrocytes.

### *Pregnancy*

Anti-Rh agglutinins were detected in the sera of 105 Rh-negative women who had been delivered of an Rh-positive infant. In 79 of these the immune response was studied in detail. In the remaining

26, a sample of maternal serum was not obtained until after the 30th day following delivery, so that, although it was possible to demonstrate immune anti-Rh agglutinins in the serum, the information with regard to the time and magnitude of the response was incomplete.



agglutinins in the maternal serum in these 5 cases makes it very likely that 3 stillbirths were in fact due to hæmolytic disease of the foetus and that the 2 cases of physiological jaundice might have been more correctly diagnosed as very mild icterus gravis neonatorum. The time of attainment of the peak titre is shown in fig. 4b. In 29 cases this was between 10 and 20 days, in 27 between 5 and 9 days, in 14 between 1 and 4 days and in 9 more than 20 days after delivery.

In 9 families in which a previous infant had been affected with hæmolytic disease, the titre of the anti-Rh iso-agglutinins in the maternal serum was estimated at intervals during a subsequent

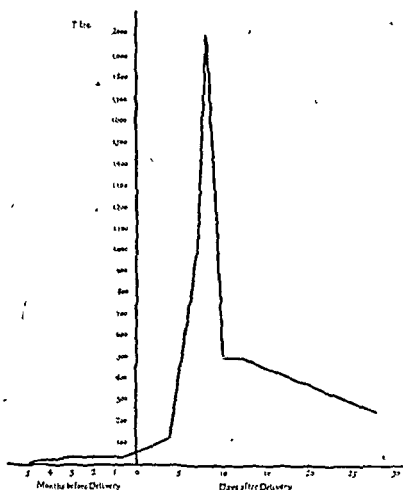


FIG. 5.—Response of the anti-Rh iso-agglutinin in an Rh-negative mother to the Rh agglutinin of the foetus during and after pregnancy.

pregnancy. In 2 cases there was an increase in the titre of the anti-Rh iso-agglutinin during pregnancy. In each of these an Rh-positive infant affected with hæmolytic disease was born and the peak of the immune response occurred in the first week after delivery. One of these is illustrated in fig. 5. In the other 7 cases there was no increase in the titre of the antibodies during pregnancy or after delivery; 5 of the infants were Rh-positive and affected with hæmolytic disease, the other two were Rh-negative and normal.

In 77 of the 79 cases no direct correlation was found between the peak titre attained and the number of stimuli received by the mother. (In the remaining 2 cases no previous history of the family was obtainable.) This was true whether all the infants or only those

affected with hæmolytic disease were considered as providing a stimulus. The lack of correlation is illustrated by fig. 6, where the families are arranged in groups according to the titre of the Rh antibodies in the mother's serum, and the children in each family are arranged in chronological order. It was not possible to type the blood of all the normal children in this series. However, of those tested, all but 2 (both in the same family) were found to be Rh-positive, and it is now recognised that in families in which hæmolytic disease of the foetus occurs the great majority of the siblings are Rh-positive (Taylor and Race, 1944). The similarity between the group of families in which the peak titre of anti-Rh agglutinins in the mother's serum was only 2 and that in which it was 256 is a very striking example

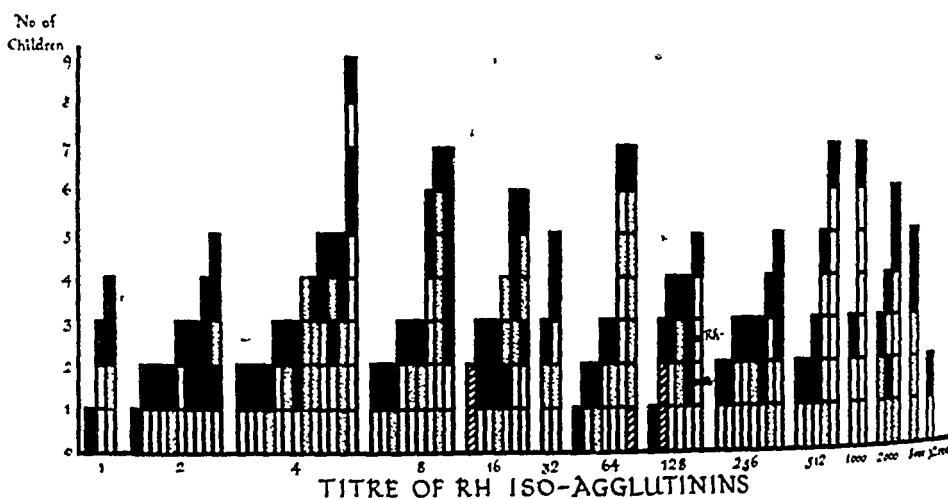


FIG. 6.—Summary of 77 families in which an anti-Rh iso-agglutinin was produced in the maternal serum showing (a) number of children in family and their clinical condition at birth, (b) the peak titre of the anti-Rh iso-agglutinin after the birth of the last infant.

of the fact that the titre of the antibody in the maternal serum is not directly related to the number of stimuli received. Another good example of this is provided by the family in which the most potent antibody (titre 32,000) was found, and in which there were only 2 children, the first normal, the second affected with hæmolytic disease.

Fig. 6 also illustrates the well known facts that in families in which hæmolytic disease of the foetus occurs the first child is usually unaffected, and that once an affected child has been born subsequent children are very likely also to be affected. Of the 77 first children in these families only four were definitely affected with hæmolytic disease. In a previous paper (Boorman *et al.*, 1944) it was mentioned that out of 100 families in which hæmolytic disease occurred the first infant was definitely affected in 10 instances, but in only 7 of these was the affection due to the formation by the mother of anti-Rh

agglutinins. In 13 other instances the first infant failed to survive, and it is possible that in a few of these hæmolytic disease may have been responsible. However, no serological tests were made until further pregnancies had occurred and definitely affected infants had been born. Thus although only an approximate figure can be given, it seems probable that in only about 10 per cent. of cases in which anti-Rh agglutinins are produced are they a response to a first pregnancy.

### *Blood transfusion and pregnancy*

A group of 7 Rh-negative women who received an Rh-positive blood transfusion just prior to or soon after delivery of an Rh-positive infant were studied. These are of interest because the immune agglutinins were produced in response to a double stimulus (Rh-positive foetus and Rh-positive blood transfusion). In 5 cases the transfused erythrocytes were eliminated within a week of transfusion, in one case within 12 days and in the other within 32-45 days. In every case anti-Rh agglutinins were demonstrated in the patient's serum after the transfused erythrocytes had been eliminated, *e.g.* in the last case mentioned they were first demonstrated on the forty-fifth day after transfusion (forty-second day after delivery). It should be noted that this was the patient's first pregnancy and that her twin infants (Rh-positive) were normal.

### *Thermal amplitude of anti-Rh iso-agglutinins*

The earliest reports of human immune anti-Rh agglutinins (Wiener and Peters) describe them as "cold" agglutinins. Levine *et al.*, however, reported that the Rh antibodies in the sera of women recently delivered of infants affected with hæmolytic disease were more active at 37° C. than at lower temperatures. We have titrated the antibodies of 61 individuals at room temperature and at 37° C. In 58 of these the anti-Rh agglutinins had been stimulated by an Rh-positive foetus and in three by transfusions of Rh-positive blood. In every instance the titre was found to be as great or greater at 37° C than at room temperature (table II).

TABLE II  
Comparison of titration values of 61 anti-Rh sera  
at room temperature and at 37° C.

	Weaker at 37° C than at room temperature	The same at 37° C as at room temperature	Stronger at 37° C than at room temperature				
No. of sera . . . . .	0	13	26	15	4	2	1
No. of times titre at 37° C. > at room temperature	0	0	2	4	8	16	32



## BONE FORMATION IN APICAL SCARS

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(PLATES XX-XXII)

FOR a considerable time morbid anatomists have differed as to whether the anthracotic subpleural apical pulmonary scars so commonly found at autopsy are the result of tuberculosis

Thus Nageli (1900) admitted that it was not always possible to be certain of their tuberculous ætiology, but thought that when calcification or caseation was present within the scars no doubt existed. Since then Focke (1924), Loeschke (1928), Huebschmann (1929), Anders (1929), Aschoff (1929), Oliva (1933-34) and Sato (1934) have expressed the belief that all apical scars are to be taken as providing evidence of previous tuberculosis (re infection lesions). Schurmann (1929), Bohne (1930), Wurm (1932) and Rubinstein and Triuss (1934), on the other hand, have upheld a non tuberculous origin for those in which foci of caseation or calcification were lacking. More recently Davson and Susman (1937) have shown that the development of these scars was closely related to the accumulation of siliceous dust at the apex and confirmed the finding of many previous workers that in most cases definite evidence of tuberculosis such as caseous or calcified foci was lacking. Their results have been confirmed by Roulet (1939), who, by chemical analyses of lung apices, showed that a definite increase in siliceous dust occurred with increasing age. Furthermore, Davson (1939), studying apices from cases aged 35 years and under, showed that the earliest evidence of apical scar formation could be seen in the second decade, and traced a gradual increase in size of the lesions with advancing age. At no stage was there evidence of active tuberculosis except when generalised tuberculosis coexisted.

On these results it seems improbable that tuberculosis plays any part in the ætiology of the majority of apical scars. The position is less clear, however, when they contain areas of calcification or ossification.

That calcified foci within the lungs might be regarded as evidence of healed tuberculosis has been recognised since Laennec (quoted by Schurmann, 1926), and that such calcified lesions may become ossified has been shown by Pollak (1901), Poscharissky (1905) and others. Hence the occurrence of either calcification or ossification within an apical scar will suggest, often correctly, previous tuberculosis. But calcification and ossification may occur in other parts of the body, e.g. in arteries (Mönckeberg, 1902), muscles (von Dittich, 1926), dura mater (Poscharissky, 1905) etc., when no suspicion of a tuberculous ætiology arises. The possibility therefore exists that even within apical scars calcification and ossification are not always due to previous tuberculosis.



This possibility is supported by the occasional finding of multiple foci of ossification in the lower lobes of elderly subjects, first described by Luschka (1856) and later by Nelius (1921), Seemann (1925), Brackertz (1929), Daust (1929), Hiebaum (1934), Kernau (1936), Amorim (1936), Teufel (1936;37) and Wells and Dunlap (1943). While favouring a chronic inflammatory or interstitial origin for these lesions, none of these authors believed that tuberculosis was the cause.

Thus the occurrence of either calcification or ossification within an apical scar need not necessarily signify previous tuberculosis, and in this paper an analysis of the incidence of these lesions in a series of apical scars is given and their relationship to tuberculosis is discussed.

#### MATERIAL AND METHODS

The material comprised the apices of the lungs from 130 nearly consecutive autopsies performed at the Manchester Royal Infirmary. In every case the apices of both lungs were cut into thin slices after formol fixation and two or more blocks of tissue from each apex were embedded in paraffin and examined histologically. Further blocks were taken of all caseous or calcified foci present in or near the apex. Decalcification was performed when necessary in 5 per cent. nitric acid. Sections were stained by hæmalum and eosin, and by Weigert's elastic and van Gieson combination.

#### RESULTS

In general the naked eye and histological findings in this series of apical scars correspond with those of the previous series described by Davson and Susman and divided by them into type A scars, in which definite evidence of tuberculosis was lacking, and type B scars, which presented evidence of tuberculosis. Naked eye and histological descriptions are therefore given only of those apical scars in which calcification or ossification was found to be present.

##### *Type A scars*

In 12 out of the 14 type A scars which showed ossification or calcification histologically there was no naked eye evidence of either. In the remaining two, both from the same case, there were present in the subpleural scar tissue numerous rather ill-defined translucent gritty foci of small size without evidence of fibrous encapsulation.

*Histology.* The essential features consisted of fibrosis of the immediately subpleural lung parenchyma together with varying degrees of carbon and siliceous dust accumulation. Elastic stains showed an intact and indeed a proliferated elastic fibre network within the entire area of the scar, corresponding in outline with the original alveolar elastic network of the lung. Caseation or encapsulated areas of calcified caseous tissue were absent. In the 12 scars in which patchy calcification was only discovered on microscopic examination hæmalum and eosin-stained sections showed that it occurred in the form of aggregations of fine bluish granules which gradually diminished in

## BONE FORMATION IN APICAL SCARS



FIG 1 — Calcified focus in a type A scar  
H and E  $\times 75$



FIG 2 — Consecutive section to fig 1, showing that the paler staining elastic network within the calcified focus is continuous with the darker staining network of the rest of the scar. Weigert's elastic stain and van Gieson  $\times 75$ .

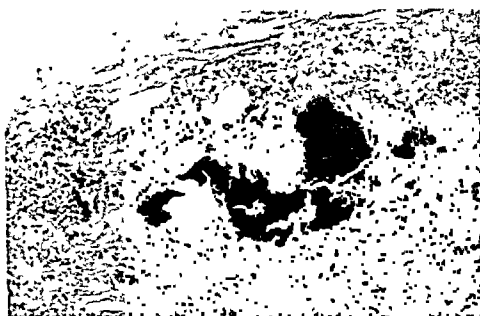


FIG 3 — Calcified area within a type A scar. H and E.  $\times 75$

FIG 4 — Adjacent section to fig 3, showing the elastic network within the calcified zone. Weigert's elastic stain and van Gieson  $\times 75$ .





number towards the periphery of the deposit (figs. 1 and 3). The calcified foci showed no definite collagenous fibrous tissue capsules and no caseation. Absence of caseation was confirmed by Weigert's elastin stain, which demonstrated the elastic network of the scar extending throughout the calcified zone (figs. 2 and 4). In two cases, however, the elastic fibres within the calcified zone stained less clearly than the remainder of the network of which they formed part. In two others lesions the calcified foci were very small and were confined to areas of collagenous fibrosis within the elastic network.

Ossification was found in relation to areas of calcification in 6 scars, and in 2, both from the same case, in which no associated calcification could be determined. Bone formation was usually easily recognised in the hæmalum and eosin-stained sections. The structure was commonly that of woven bone, but where central bone marrow formation had occurred (fig. 8) lamellar bone could be seen to be present in close relation to the marrow spaces. A collagenous fibrous capsule was lacking around the bony foci (figs. 5-8) and there was no central calcareous nor caseous mass, the bone often being continuous with a calcified or non-calcified area of fibrous tissue forming part of the main apical scar. Weigert's elastin stain showed that the proliferated intact elastic network of the scarred area coursed up to and often through the area of bone (figs. 6, 7 and 9). There was no definite evidence of osteoclastic resorption of the calcified fibrous tissue and it was not clear how the calcified fibrous tissue became converted into bone, but the presence of intact elastic fibres within the bone substance suggests that no massive osteoclastic resorption had preceded bone formation. However, where bone marrow spaces were present, evidence of lacunar absorption of woven bone was seen and, adjacent to such areas, lamellar bone had been laid down. Moreover in such lamellar bone elastic fibrils were for the most part absent, having probably been removed when the woven bone underwent osteoclastic resorption prior to the deposition of lamellar bone.

*Age incidence.* The ages of the 6 cases of ossification in type A scars ranged between 62 and 78 (average 70 years).

#### *Type B scars*

Of 16 type B apical scars in which evidence of healed tuberculosis was found on subsequent histological examination, 13 showed calcification, and of these, 7 (from 6 cases) showed various degrees of ossification, while 3 scars showed fibrous nodules which were regarded as probably tuberculous.

*Naked eye appearances.* In 10 of the scars the lesions were immediately subpleural in site, in the remaining 6 they were somewhat deeper within the apex. On section the lesions consisted of small hard pale yellow foci surrounded by well defined greyish rings of fibrous tissue, around which were ill-defined areas of anthracotic

lung tissue of varying amount. In the 3 scars that contained fibrous nodules the lesions consisted of firm greyish white foci of small size.

*Histology.* The yellowish foci consisted of central masses of calcified necrotic tissue surrounded by a collagen fibre capsule that clearly demarcated the calcified mass from the surrounding lung tissue. This in turn showed irregular fibrosis in varying amount, together with carbon pigment accumulation. Bone formation in some degree was present in 7 scars and was usually of lamellar type, being laid down in an incomplete ring between the fibrous capsule and the central calcified mass. Howship's lacunæ along the edge of the calcified mass were frequently seen, and in two lesions osteoclasts were seen lying between the calcified mass and the fibrous tissue capsule. Thus in type B scars there is definite evidence that osteoclastic resorption precedes bone formation.

Elastic staining showed that elastic fibres were either absent from the calcified areas, or, as in 2 cases, were fragmented and thin and did not form part of a network extending in from the surrounding lung (fig. 10). The proliferated elastic network of the surrounding lung tissue was sharply demarcated from the central calcified area by a zone of dense fibrous tissue in which no elastic fibres were present (fig. 10), or if visible, existed only as isolated fragments and not as part of a continuous network such as was seen in the calcified or ossified areas of type A scars.

Bone marrow formation was recognised in relation to bone formation in 3 scars.

*Age incidence.* The ages of the 7 cases showing ossification in type B scars ranged between 48 and 66 (average 57 years).

#### *Summary of the differences between type A and type B scars*

(1) Areas of calcification and ossification are usually recognisable to the naked eye in type B scars, in type A only as a rule on histological examination. (2) Calcification in type B primarily involves a necrotic mass; in type A there is no obvious necrosis, calcification occurring in small irregular deposits in the fibrous tissue of the scar. (3) Bone formation in type B begins at the periphery of the main calcified mass; in type A it is haphazard in its relation to the foci of calcification and may apparently develop without previous calcification of fibrous tissue. (4) The elastic network is fragmentary or absent in type B areas of calcification or bone; in type A lesions the intact elastic network can be demonstrated within the area of calcification or ossification in direct continuity with the network outside.

#### DISCUSSION

Ossification and calcification within apical scars may be the result of two distinct processes. They may occur as the later stages of the

## BONE FORMATION IN ALICAL SCARS



FIG. 7—A larger focus of woven bone within a type A scar, showing the intact elastic network. Weigert's elastic stain and van Gieson.  $\times 70$ .



FIG. 10—Calcification and ossification in a type B scar. Elastic fibres can only be seen external to the thick collagen fibre capsule which separates the calcified caseous tissue from the surrounding lung parenchyma. Weigert's elastic stain and van Gieson.  $\times 70$ .



FIG. 8—Bone and bone marrow formation within a type A scar. Weigert's elastic stain and van Gieson.  $\times 75$ .



FIG. 9—Higher magnification of the bone in fig. 8 showing the elastic fibres within the bone. Weigert's elastic stain and van Gieson.  $\times 160$ .



healing process in encapsulated areas of tuberculous caseation, *i.e.* in type B scars, or they may develop as late phenomena within type A (non-tuberculous) scars. To a certain extent this view suggests that previous work on the incidence of healed apical tuberculosis (so-called reinfection lesions) may require slight modification. Thus Nägeli (1900), and Burkhardt (1906-07) assumed that the presence of calcification within apical scars was evidence of previous tuberculosis, but neither discussed the significance of bone formation. Opie (1917) may have interpreted such non-specific calcification and ossification as evidence of healed tuberculosis in his radiological studies of autopsy lungs, since he does not always seem to have confirmed his results histologically. However, as these non-specific lesions are found only in the later age-groups (50-80 years), they are not likely to have affected materially the validity of his figures. Terplan (1940), in his recent series of papers on the morbid anatomy of pulmonary tuberculosis, does not mention type A scars nor the possible significance of calcification or ossification within them, so that it is not possible to be certain whether he attached any importance to their presence in arriving at the figure of 97 per cent. for the incidence of tuberculous lesions in 99 autopsies between the ages of 50 and 90 years.

Ossification in the lower lobes as a rare autopsy finding has been described, as mentioned above, by Luschka and others. Most authors (Nelius, Seemann, Daust, Hiebaum, Kernau, Teuffl) believe that the process of ossification occurs by direct change of either fibrous tissue or an inflammatory exudate into bone without previous osteoclastic resorption of a calcified matrix, and several (Nelius, Daust, Kernau) describe the presence of intact elastic fibres of the alveolar network within the areas of bone. The parallelism between their observations and the findings in this study of bone formation in type A scars suggests that a similar mechanism is involved which is different from the mode of ossification in type B scars, where osteoclastic resorption of a calcified matrix precedes bone deposition (Pollak, 1901; Poscharissky, 1905; Wurm, 1926; Schürmann, 1926).

Thus in type A scars the first stage of ossification consists of the laying down of woven bone, either in association with a focus of calcified scar tissue or in fibrous tissue not previously calcified. The second stage, when present, consists of osteoclastic resorption of the woven bone with formation of a bone marrow space and subsequent laying down of lamellar bone on the surface of the eroded woven bone.

In type B scars, on the other hand, the calcified tissue undergoes osteoclastic resorption directly, a bone marrow space forms and lamellar bone is laid down on the surface of the calcified tissue.

The presence of intact elastic fibres within bone was first described as occurring in normal bone by Muller (1860) and later by Schafer (1878) (both quoted by Weidenreich, 1930), so that their occurrence in heterotopic bone is not an isolated pathological phenomenon.



The average age of the 6 cases in which ossification in type A scars occurred was 70 years, while that of the 6 cases in which ossification in type B scars occurred was 57 years. This age difference is statistically significant and it may be concluded therefore that ossification is a somewhat later phenomenon in type A than in type B scars. It is interesting to note that Daust observed that the average age for the Luschka type of lower lobe ossification was  $67\frac{1}{2}$  years, corresponding with the figure of 70 years for type A scars in this series.

### SUMMARY

1. The apices of the lungs from 130 autopsies were studied histologically.

2. Calcification alone was present in 6 out of 14 type A (non-tuberculous) scars, calcification and ossification in 6 and ossification alone in 2. In 12 of these scars there was no naked eye evidence of either calcification or ossification.

3. Calcification was found in 13 out of 16 type B (tuberculous) scars and of these, 7 showed ossification.

4. The presence of an intact elastic network within calcified and ossified areas in type A scars and its absence in type B lesions is demonstrated.

5. The mechanism of ossification in type A scars resembles that occurring in the Luschka type of pulmonary ossification and differs from the mechanism occurring in type B scars.

6. Ossification occurs at a later age in type A than in type B scars.

7. Calcification and ossification in apical scars are not always indicative of previous tuberculosis.

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## THE ANTIGENIC STRUCTURE OF ERYSIPELOTHRIX

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SWINE erysipelas caused by *Erysipelothrix rhusiopathiae* occasions considerable losses amongst pigs in most countries of the world and apparently identical organisms have been held responsible for disease in man and in many species of animals. It is therefore of considerable importance to determine whether there exist differences between strains from different hosts and from various parts of the world. From the biochemical standpoint the group is practically homogeneous and such differences as exist are too variable to provide a basis for classification (Watts, 1940; Karlson and Merchant, 1941). As regards serological classification the literature shows that all strains are agglutinated by immune sera prepared against pig strains, so that there must be at least one common antigen. There is, however, no agreement as to whether the group is homogeneous. In his study of 43 strains obtained from many countries and from various species of animals, Watts confirmed that cross agglutination occurs between all strains; he demonstrated the existence of two group-specific heat-stable antigens capable of withstanding boiling for four hours, which enabled him to divide his strains into two groups, 38 belonging to one and 5 to the other. In order to explain why cross agglutination occurs between the organisms of these two groups and why sera prepared from antigens of one group will give a high degree of protection only to mice infected with the same group, he suggested that each probably contains two heat-labile antigens present in different proportions in the two groups. Atkinson (1941), however, claims that there are intermediate strains in addition to two well marked groups, while Julianelle (1941) found the strains he studied to be homogeneous.

## TECHNIQUE

*Maintenance of cultures.* All strains used in this investigation were smooth and were maintained by growing them on 3-5 per cent. horse serum broth, subculturing once a month.

*Preparation of suspensions.* Strains were subcultured daily on the serum-broth medium before inoculating bottles of glucose broth of pH 7.8. These were incubated at 37° C. for 24 hours, centrifuged and the bacterial deposit re-suspended in normal saline containing 1:2000 merthiolate. Such suspensions, of a concentration 5-10 times Brown's tube no. 10, were stored in the cold room and diluted with normal saline as required. The method was not satisfactory

for the production of large amounts of antigen owing to the poor growth and the large volumes which required to be centrifuged. Other cultural methods were tried without success.

*Preparation of antisera.* Following Watts's method, doses of 1, 2, 3 and 3 c.c. of antigen were injected intravenously into rabbits, the opacity being standardised to twice that of Brown's tube no. 10. The injections were at 4-day intervals and the rabbits were bled 10 days after the last injection. In the preparation of antisera with boiled antigens, suspensions were boiled for 4 hours before injection.

*Method of carrying out the agglutination test.* Merthiolated antigen of an opacity equal to Brown's tube no. 3 was added to equal volumes of twofold dilutions of serum, the final proportion of serum in the first tube being 1:20. The tubes were incubated at 42° C. in the water-bath for 4 hours and at 37° C. in the incubator for a further 14 hours, making 18 hours in all. The results, recorded at 4 hours and 18 hours, were signified by + for very slight agglutination to ++++ for complete agglutination. The titres of sera in the following tables have been taken as the last tube showing +++ agglutination after 18 hours. This procedure was adopted as it often happened that sera showed very nearly complete agglutination over a wide range, while in few tubes was it absolutely complete. Unless explicitly stated, boiled suspensions were not used as antigen for agglutination tests because they tended to be unstable.

*Absorption of sera.* The sera were mixed with suspensions to give a dilution of 1:5 and incubated at 37° C. After agglutination and centrifuging, the dilution of the supernatant was increased to 1:10 by adding more antigen. Further amounts of the stock antigen were centrifuged as required to complete the absorption, the deposits being re-suspended in the diluted serum so that the dilution was maintained at 1:10. The absorption was deemed to be complete when the serum could no longer clear itself of antigen. Absorption of agglutinins often took a fortnight, though it was quicker when the antigen employed could not completely absorb the serum. Where absorption was incomplete, incubation at 37° C. in the presence of antigen was continued for a fortnight in order to ensure that the long period of incubation of itself did no appreciable damage to the agglutinins. This procedure for absorption was followed in preference to the more usual single addition of antigen in excess in order to economise in antigen, which was difficult to produce in large amounts.

## RESULTS

### *The heat stability of antigens of Erysipelothrix*

Watts's results were taken as a starting point in the present work so that it was necessary, in the first place, to prepare specific sera against boiled antigens. For this purpose 11 cultures were selected, 6 of which were obtained from Watts and 2 from the National Collection of Type Cultures, the remaining 3 being isolated by the writer from a pig, a duck and a mouse. Of Watts's cultures 3 were known to belong to his rarer group and from 2 of these sera were prepared in rabbits. Sera were also prepared from 2 of the remaining 8, the antigen being boiled for 4 hours in all cases. The results obtained with these 4 antisera and the 11 strains are shown in table I. It will be seen that with one serum of each of Watts's groups, namely D and EW 2, considerable cross agglutination occurs. This is not in agreement with the group specificity of the heat-stable antigens claimed by him. It is evident

that either the heat-stable antigens are not entirely group-specific or the common heat-labile antigens survive boiling to a sufficient extent to produce antibodies when injected into rabbits in the large doses used. In this case, boiled antigen might not be expected to be agglutinated by heterologous sera, for if the heat-labile antigen had escaped destruction on the majority of the bacterial cells, there are no grounds for calling it thermolabile at all. To test this point, the titres of sera prepared with EW 2 unboiled and EW 6 unboiled were determined with boiled antigens of EW 2, EW 6, EW 11 and MEW 22. The titres of both sera were 1:640 with boiled homologous antigen

TABLE I

*Titres of sera prepared with boiled antigen against  
11 selected strains of Erysipelothrix*

Antigen	Serum prepared against strain			
	R boiled	D boiled	EW 2 boiled	EW 10 boiled
EW 2	0	320	2560	2560
EW 10	0	160	640	1280
EW 24	0	80	1280	320
EW 11	0	40	640	80
R	80	640	320	20
D	160	5120	320	20
EW 6	640	640	640	40
EW 9	160	160	320	0
1224	640	1280	640	40
3406	640	320	320	20
MEW 22	0	160	640	80

EW 2, EW 10 and EW 24 correspond to Watts's rarer group.

0 = no agglutination in a serum dilution of 1:20.

and 1:160 with boiled heterologous antigens except EW 11, with which the titres were 1:80. From this it is inferred that the heat-stable antigens are not exclusively specific. It is therefore unnecessary to postulate the existence of thermolabile antigens to explain the cross agglutination which occurs between sera and heterologous strains. Further evidence on this point was sought by absorbing each of four sera prepared against unboiled suspensions of EW 2, EW 6, EW 11 and MEW 22 with boiled suspensions of all of these strains. In this way all agglutinins corresponding to thermostable antigens were removed in each case. The resulting sera failed to agglutinate unboiled suspensions of these strains, which provides strong evidence that at any rate these four strains contain no heat-labile antigens.

#### *Classification of strains according to heat-stable antigens*

As there is no reason for postulating the existence of heat-labile agglutinogens, there must be at least one common heat-stable antigen to account for cross agglutination. To determine whether there are

in addition specific heat-stable antigens, sera were absorbed by heterologous strains and examined for residual agglutinins. (It should perhaps be stated that the term antigen refers to reactive groups, all of which may be components of one antigen in the more correct sense; the term is, however, retained since it leads to no ambiguity here.)

The two sera of table I prepared against D boiled and EW 10 boiled were absorbed by the heterologous strains EW 2 and R respectively. Of the remaining two sera shown in table I, that prepared against R boiled required no absorption to reveal its group-specific character, while that prepared against EW 2 boiled was absorbed and used against a larger series of antigens later (table III). It will be seen in table II that 9 of the antigens fall into two groups with no significant cross agglutination, while EW 11 and MEW 22 belong to neither group. Neither of these antigens was agglutinated by a mixture of absorbed sera of each group. Sera were prepared from them and it was found after absorption that each formed a separate group although both sera required to be absorbed with strains of both of Watts's groups before their group specificity became manifest.

TABLE II  
*Agglutination titres of absorbed sera*

Antigen	Sera prepared against strain	
	D boiled, absorbed with EW 2	EW 10 boiled, absorbed with R
EW 2	0	1280
EW 10	0	320
EW 24	0	320
EW 11	0	20
R	160	0
D	640	0
EW 6	640	0
EW 9	80	0
1224	320	0
3406	160	0
MEW 22	0	0

Following the classification of the eleven cultures into four groups, other strains were tested against absorbed sera of these groups. Twenty further strains, of which 12 were isolated from pigs, 4 from sheep, 2 from mice, one from a goose and one from an African jacana, were obtained from Dr R. F. Montgomerie, Dr P. S. Watts, Miss N. Atkinson and the Curator of the National Collection of Type Cultures. The results with these strains, together with the 4 type strains EW 6, EW 2, EW 11 and MEW 22, are shown in table III.

From the results in tables I-III, 24 of 31 strains fall into groups as under.

Group I (EW 6, R, D, EW 9, 1224, 3406, 1694, Ru, HSE 2)	9 strains
Group II (EW 2, EW 10, EW 24, PLK, 2422)	5 "
Group III (EW 11, EW 16, HE, Ely, V)	5 "
Group IV (MEW 22)	1 strain
Agglutinated by both group I and group II sera (PLA, AE 1, AE 2 and AE 5)	4 strains

The remaining 7 strains (5926, 1414, 2420, 807 B, 3260, 2825 and 3259) were not agglutinated by any of the absorbed sera nor by a mixture of these sera, so that it was evident that they formed at least one additional group. Sera were prepared from the boiled

TABLE III

*Titres of the four group-specific sera with antigens of the twenty additional strains (one strain typical of each group being included for comparison)*

Antigens	Serum prepared against			
	group I R boiled, not absorbed	group II EW 2 boiled, absorbed R(I) and EW 11 (III)	group III EW 11 boiled, absorbed EW 2 (II) and EW 6 (I)	group IV MEW 22 boiled, absorbed R (I) and EW 11 (III)
EW 6 (I)	160	0	0	0
EW 2 (II)	0	1280	0	0
EW 11 (III)	0	0	160	0
MEW 22 (IV)	0	0	0	160
Ru	40	0	0	0
V	0	0	80	0
PLK	0	320	0	0
EW 16	0	0	160	0
1694	80	0	0	0
2422	0	160	0	0
HSE 2	80	0	0	0
HE	0	0	40	0
Ely	0	0	40	0
5926	0	0	0	0
1414	0	0	0	0
2420	0	0	0	0
807 B	0	0	0	0
3260	0	0	0	0
2825	0	0	0	0
3259	0	0	0	0
PLA	160	320	0	0
AE 1	40	80	0	0
AE 2	160	320	0	0
AE 5	160	640	0	0

In this and subsequent tables the Roman numerals in brackets indicate the serological groups to which strains belong.

antigen of two of them (3260 and 1414). The titres of these two sera with the homologous strains were 1:160 and 1:80 respectively and with the four type strains varied between 1:40 and 1:320. No attempt was made to produce type-specific sera by absorption because, owing to the low titres with homologous strains, complete absorption would almost certainly have occurred. No attempt was made to produce specific sera with the remainder of these 7 strains, since the classification had become impracticable because of these strains and the four agglutinated by specific sera of both group I and group II.



*The distribution of heat-stable antigens in strains of  
different serological groups*

The simplest antigenic structure to account for the results obtained is to postulate a common heat-stable antigen to explain cross agglutination and, in addition, at least five specific heat-stable antigens to account for the four groups and the seven aberrant strains just considered. For the results with the last four strains in table III, there would appear to be two explanations consistent with this hypothesis. Either these strains possess antigens of groups I and II and were therefore agglutinated by mono-specific sera of both groups, or the sera used for grouping in table III were not mono-specific. That the latter alternative is probably correct was shown by absorbing the group II serum used for the tests of table III, in one instance with AE 1 and in the other with AE 2. The titres of these absorbed sera with all the group II strains are shown in table IV. It will be

TABLE IV

*Agglutination titres of serum prepared with EW 2 boiled*

Antigens	Serum prepared against strain EW 2 boiled and absorbed with	
	R, EW 11 and AE 1	R, EW 11 and AE 2
EW 2 (II)	160	80
EW 10 (II)	160	160
EW 24 (II)	40	40
PLK (II)	40	40
2422 (II)	20	40
PLA	...	0
AE 1	0	0
AE 2	0	0
AE 5	0	0

seen that the four strains agglutinated by group I and group II sera do not belong to group II. Also, the lower titres of EW 24, PLK and 2422 as compared with EW 2 and EW 10 give the impression that further absorption would differentiate them into a separate group. However that may be, AE 1, AE 2 and AE 5 do not themselves form a single group, as was shown by the fact that AE 2 alone was agglutinated by a serum prepared against strain 5926 and absorbed with EW 2, EW 11 and MEW 22. It should be stated that AE 1 and AE 2 completely absorbed the group I specific serum, so that these strains contain group I antigen. It is concluded from these results that specific antigens may be shared to some extent by strains of other groups. That serological grouping is possible with many strains, however, does suggest that for them there is one major agglutinin in addition to the common agglutinin and the results with strains AE 1, AE 2, AE 5 and PLA are not inconsistent with this view, since they are not agglutinated by truly specific group II serum.

*Absorption experiments with sera prepared against unboiled antigen*

The results of five absorptions with heterologous strains of sera prepared against unboiled antigen are given in table V; in three of

TABLE V

*Absorption of sera prepared from unboiled antigen by heterologous strains*

Serum prepared against	Titre with homologous strain before absorption	Absorbing strain	Titre with homologous strain after absorption
EW 2 (II)	2560	R (I)	0
EW 2 (II)	2560	EW 11 (III)	0
R (I)	640	EW 2 (II)	80
R (I)	640	EW 10 (II)	80
EW 6 (I)	5120	EW 2 (II)	0

the tests complete absorption occurred. In contrast, it has not been found possible by the method employed to absorb completely sera prepared with boiled antigen. This difference is explained by the hypothesis that in sera prepared against unboiled antigen the heterologous agglutinins are in relatively greater concentration. Much greater amounts of absorbing antigen were required before reaching the point at which the absorbing strain was no longer agglutinated; in consequence of this agglutinins which were minor in relation to the absorbing strain were absorbed to a correspondingly greater degree. If this explanation is correct, it should be possible to absorb with a heterologous strain which cannot normally absorb it all agglutinins from a serum prepared against boiled antigen by increasing in the serum prior to absorption the amount of agglutinins corresponding to the common antigen and to the absorbing strain's specific antigen.

This was achieved by adding serum prepared against the heterologous absorbing strain to the serum to be absorbed. The absorption technique did not differ from that described; the only point to be noted was that the final serum dilution of 1:10 of the former technique refers here to the serum to be absorbed only, the serum corresponding to the absorbing strain being ignored. The method employed for the absorption of group I serum prepared against boiled antigen with a group II antigen, and *vice versa*, required some adjustment of the dose of added serum and in one case the addition of a group III specific serum before complete absorption resulted.

Unless the absorption be attributed to some non-specific phenomenon, the hypothesis which suggested the experiment receives confirmation. The method was also extended with success to the two sera prepared against unboiled antigen which were not absorbed in table V; the results with sera against boiled and unboiled antigen are shown in table VI.

Direct confirmation that the inability of heterologous strains to

absorb sera prepared with boiled antigens was consequent upon the absorption technique adopted was obtained by absorbing sera prepared against a boiled group III strain (EW 11) and a boiled group IV strain (MEW 22) with a group I (EW 6) and group II strain (EW 2) respectively at 0° C., and continuing the additions of antigen after agglutination had ceased. The homologous titres of these sera before absorption were 1:640 and 1:2560 respectively. After absorbing

TABLE VI

*Absorption of agglutinins by heterologous strains from sera to which other sera have been added*

Serum to be absorbed prepared against strain	Titre with homologous strain before absorption	Quantity used	Sera added, prepared against strains	Quantity used	Absorbing strain	Titre with homologous strain
R boiled (I)	80	0.1 c.c.	EW 2 boiled (II)	0.5 c.c.	EW 2 (II)	0
EW 2 boiled (II)	2560	0.05 c.c.	R boiled (I)	0.25 c.c.	R (I)	0
...	...	...	EW 11 boiled (III) absorbed*	0.20 c.c.	...	...
R unboiled (I)	640	0.1 c.c.	EW 2 boiled (II)	0.1 c.c.	EW 2 (II)	0
R unboiled (I)	640	0.1 c.c.	EW 2 and EW 11 boiled (II and III)	0.1 c.c. of each	EW 10 (II)	0

\* EW 11 boiled absorbed refers to a serum prepared with EW 11 boiled and absorbed with EW 2 and EW 6 so that it was specific for the EW 11 group as shown in table III.

them with amounts of antigen equal to about three times the amount of homologous antigen which would have been required for complete absorption, the serum against EW 11 boiled was absorbed while that against MEW 22 agglutinated this strain completely at a dilution of 1:20 and partially at 1:40. This serum was completely absorbed after further addition of about half as much antigen as had been already used. These findings suggest that the absorbing strain possesses, as a minor antigen, the major antigen of the strain with which the serum was prepared. Though few absorptions of this kind were carried out, absorbing strains were selected as being least likely to absorb sera on the basis of the classification of table III.

## DISCUSSION

The results of the experiments described might be explained on the hypothesis that the antigens are heat-stable and that each strain possesses one which is common to all strains and one which is specific. In addition, strains possess as minor antigens the specific antigens of many other strains, in such a way or in such amount that their interaction with corresponding agglutinins does not result in agglutination. In view of the fact that complete absorption of sera with heterologous strains was achieved in the experiments undertaken, it is unnecessary to postulate a common antigen to explain cross agglutination if, instead, it is assumed that strains possess most

antigens in some quantity or arrangement. Apart from the greater number of antigens, the antigenic structure of the *Erysipelothrix* group according to this hypothesis resembles that ascribed to the *Brucella* group by Wilson and Miles (1932). That strains of *Brucella* should absorb minor agglutinins rather easily while strains of *Erysipelothrix* absorb them only with difficulty would not be unexpected, since the quantity of the single minor antigen on bacterial cells of the former group would greatly exceed that of any one of the many minor antigens on cells of the latter group; a larger number of *Erysipelothrix* organisms would therefore be required to absorb a heterologous serum than in the case of *Brucella* organisms. If, however, the existence of a common antigen is maintained, then there is a general resemblance to the *Brucella* group among the specific antigens. The facts elucidated in the *Erysipelothrix* group are of the same kind as in the *Brucella* group, although they have been determined in relation to fewer strains.

To account for the differences between sera prepared with unboiled and boiled antigen, it must be assumed that, though the antigens are heat stable, they suffer some damage by prolonged boiling. Sera prepared with boiled antigen tend to be more specific and are more difficult to absorb with heterologous strains. Assuming a common antigen, the explanation would be that this antigen is somewhat less heat-stable than the specific antigens, so that it is reduced in quantity in the boiled suspensions used for rabbit immunisation, with the result that the agglutinins corresponding to it would be produced less abundantly. This explanation is not easy to reconcile with the fact that boiled suspensions may produce sera which are practically specific or sera which agglutinate heterologous strains at considerable dilutions (see table I). On the hypothesis that there is no common antigen, the differences are explained by supposing that prolonged boiling reduces the antigenic content of a suspension, but the amount of the major antigen which withstands heat treatment is more likely than the minor antigens to stimulate antibody formation in the rabbit. That some boiled suspensions produce sera which are almost specific while others produce sera which agglutinate heterologous strains may be expected to result from comparative differences in quantity of minor antigens in relation to major antigen in different strains. The immunising power of boiled suspensions should therefore be approximately equivalent to that of unboiled suspensions in smaller doses. Some direct confirmation of this view has been obtained in tests with sera prepared with unboiled antigen in doses 1/100th of those previously used. The results with two sera prepared in this way are shown in table VII. It is evident that without absorption of any kind the sera divide the strains into two groups shown to exist previously by means of sera prepared with boiled antigens and absorbed in most cases.

These results appear to be in keeping with the work of Atkinson,

who classified strains of *Erysipelothrix* into two distinct groups and an intermediate group. She kindly supplied a culture belonging to each distinct group (AE 1 and AE 2) and one belonging to the intermediate group (AE 5). In the preparation of sera she gave eight

TABLE VII

*The agglutinating titres of sera prepared by immunising rabbits with small doses of unboiled antigen*

Serum prepared against	Antigens										
	Group I						Group II			Group III	Group IV
	EW 6	R	EW 9	1224	3408	D	EW 2	EW 10	EW 24	EW 11	MEW 22
EW 6 (I)	320	80	160	640	320	80	0	0	20	0	0
EW 2 (II)	0	0	0	0	0	0	80	320	160	0	0

graded doses on succeeding days, the final dose having a strength of about 1/20th to 1/30th of that used in the work described here. Reference to tables III and IV shows that her three strains were not separated from each other by the four absorbed sera used for grouping. There was evidence, however, that the major antigen of AE 2 differed from that of AE 1 and AE 5, which is in agreement with her findings. It would appear that her three type strains possess a closer resemblance to each other than that existing between typical strains of the groups described above. That she separated them partially by the direct agglutination technique and more completely by the indirect technique was probably due to the method she adopted in preparing sera, her results being comparable with those shown in table VII rather than those of preceding tables.

### SUMMARY

1. The antigens associated with strains of *Erysipelothrix* were shown to be heat-stable but suffered some damage by prolonged boiling, which accounts for the differences observed between sera prepared with boiled and unboiled antigens.

2. Twenty out of 31 strains were classified into four serological groups by agglutination-absorption methods. To classify the remainder, several more groups would have been required.

3. Under certain conditions sera could be completely absorbed by heterologous strains.

4. The results are compatible with an antigenic structure in which strains are qualitatively homogeneous as regards their antigens: of these there are a large number, the difference between serological groups arising from differences in the quantitative or spatial distribution of these antigens.

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# THE EFFECT OF HUMIDITY ON THE SURVIVAL OF DRIED CULTURES OF *STREPTOCOCCUS AGALACTIÆ*

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DESPITE the considerable amount of information available on the survival of streptococci and other bacteria when dried and maintained in this state (recent lists of references are given by Morton and Pulaski (1938) and by Heller (1941)), there appear to be no references to the behaviour of bacteria when maintained under other conditions of humidity. In work in progress it was necessary to preserve certain strains of *Streptococcus agalactiæ* for a considerable time. Repeated subculture was to be avoided and no apparatus for the rapid freezing and dehydration of the cultures was available. It was considered possible that as milk is the normal pabulum for this organism it might be valuable as a protective agent, since Heller has shown that, when the menstruum is colloidal, bacteria remain viable much longer.

Absolute drying of the cultures was not attempted in the present study, but they were desiccated as thin films on the inner surface of small test-tubes so that the bacteria were surrounded by a film of dried milk solids, including protein, but negligible amounts of fat—less than 0.1 per cent. of the original bulk of the milk. The majority of the cultures were stored over pure sulphuric acid (this does not fume) and were thus in a completely dry atmosphere, but advantage was taken of some spare space in a number of desiccators containing mixtures of sulphuric acid and water to store some of the cultures at humidities higher than 0 per cent. The results obtained seemed to show that the death-rate of bacteria at humidities higher than 0 per cent. did not bear a linear relationship to the humidity and that at any given humidity the death-rate was not logarithmic. The cultures stored at 0 per cent. humidity remained viable for three years and were found to be unchanged morphologically and to have the same serological and biochemical reactions as before desiccation. The experiment was therefore expanded to include more humidities and a fresh series of dried cultures was prepared, using three strains of *Str. agalactiæ* of geographically separate origin.

## *Experimental technique*

Litmus milk cultures were incubated for 18 hours and then removed and neutralised with sterile N/1 sodium hydroxide solution. Using graduated pasteur pipettes delivering 40 drops to the millilitre, a single drop of culture was placed



in each of a large number of small sterile test tubes measuring 3 by  $\frac{1}{16}$  in. These were then mounted upright in holes in wooden trays and placed in an incubator at 37° C. over  $\text{CaCl}_2$ . After 24 hours they were removed and placed in desiccators over mixtures of sulphuric acid and water of the following specific gravities: 1.84, 1.55, 1.51, 1.45, 1.4, 1.332, 1.312, 1.29, 1.27, 1.247, 1.22 and 1.00. These represent relative humidities of 0, 10, 15, 25, 35, 50, 55, 60, 65, 70, 75 and 100 per cent. The desiccators were then stored at room temperature under benches away from direct light (for the influence of this factor on the survival of bacteria see Solowey *et al.*, 1942). A quantity of uninoculated culture medium calculated to give a film of similar thickness to that of the bacterial films was also exposed in petri dishes in the desiccators and was found to have reached equilibrium with the atmosphere within 48 hours.

The moisture content of similar dried skimmed milk was also calculated by exposing quantities of 20 ml. in petri dishes to a series of humidities. Drying in the incubator over  $\text{CaCl}_2$  does not remove all the water, since further storage of these samples over  $\text{P}_2\text{O}_5$  showed a steady loss at each weighing. In view of the complexity of the substrate and the difficulty of defining when this was absolutely dry it was decided to use as the "dried weight" the weight of milk after drying over  $\text{CaCl}_2$  at 37° C. for 48 hours and storage over  $\text{P}_2\text{O}_5$  for 8 days. This treatment differs from that accorded to the actual cultures, but for the purposes of the experiment it was desired to know the absolute moisture content (so far as it could be ascertained) and therefore comparable treatment in drying was not necessary. These dried milk films were exposed to the various humidities and weighed until equilibrium was reached. The results recorded as moisture content expressed as a percentage of the "equilibrium weight" are given in the second column of the table.

Counts of viable bacteria were made before the cultures were placed in the desiccators and thereafter at varying intervals (which became longer as the experiment continued) for the duration of the experiment or for as long as viable bacteria were present, whichever was the shorter. For this the dried drop at the bottom of each tube was emulsified in one drop of sterile tap water by rubbing with a sterile glass rod with a ground end. Approximately 0.5 ml. of water was then transferred from a 10 ml. water blank to the tube and the resulting emulsion removed and returned to the same water blank. This gave a dilution of the original culture of  $\frac{1}{20}$  ( $\frac{1}{10}$  ml. in 10 ml.). Decimal dilutions in tap water were made and duplicate plates poured using 1 ml. of dilution and 10 ml. of blood agar to each plate.

### Results

Every week at first and then at longer intervals up to three months, duplicate tubes of each culture were removed from each desiccator and viable bacterial counts made; the three cultures behaved identically throughout the experiment. When the tubes were placed in the desiccators the count was  $150 \times 10^6$  per ml. After the second week the cultures at 100 per cent. relative humidity were discarded because of moulding. The results at the other humidities are shown on figs. 1 and 2, where the logarithms of the average counts of the three cultures are plotted against the time. All the curves cannot be shown on one graph owing to overcrowding; they are therefore divided into two, those over 35 per cent. humidity being regarded as in atmospheres of high humidity and those under this as in low humidities. This division is quite arbitrary and merely



convenient for recording. The graphs show that at 75 and 70 per cent. relative humidity there was no appreciable difference, all the cultures in both desiccators being dead by 10 weeks. At 65 per cent. there was a slight delay to 11 weeks before death. Below this humidity the

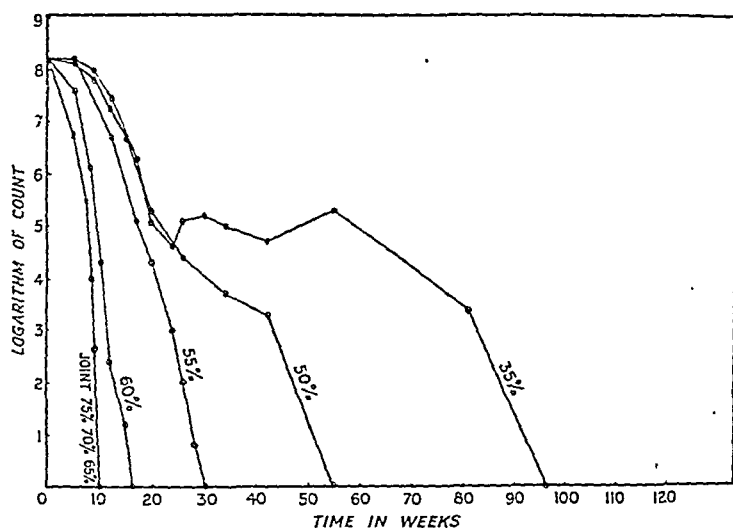


FIG. 1.—Survivor curves of *Str. agalactiae* in films of dried skimmed milk at high humidities (over 35 per cent. relative humidity).

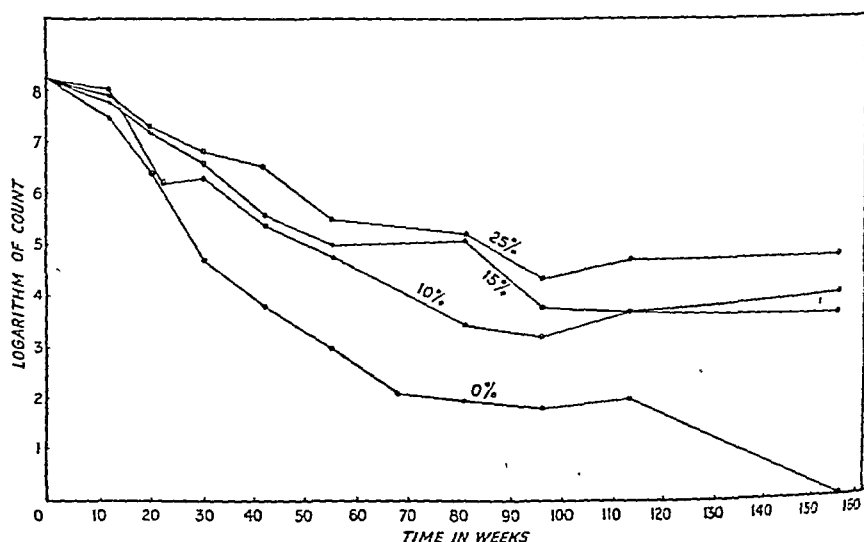


FIG. 2.—Survivor curves of *Str. agalactiae* in films of dried skimmed milk at low humidities (under 35 per cent. relative humidity).

time which elapsed before death increased rapidly. Thus at 60 per cent. it was 16 weeks, at 55 per cent. 30 weeks, at 50 per cent. 55 weeks, and at 35 per cent. 96 weeks. The next set of cultures to die were those at 0 per cent. humidity, i.e. those in the driest state. These were all dead by the 156th week, i.e. after 3 years, while the cultures

stored at 25, 15 and 10 per cent. humidity were still viable. Unfortunately the experiment had then to be discontinued as no further material was available in these desiccators. Owing to the scale at which these graphs have to be drawn, no relationship is shown between the shape of the curves at each humidity, but in fact the curve shown for 35 per cent. humidity can be regarded as more or less typical since, if suitable scales of time are chosen, the majority of the other curves approximate to this shape. That is, after a relatively short period during which the numbers are maintained at about the original level there is a fall to a definite figure at which the count is maintained until there is a fall to nil. The curves of 25, 15 and 10 per cent. humidity have not reached this stage and the

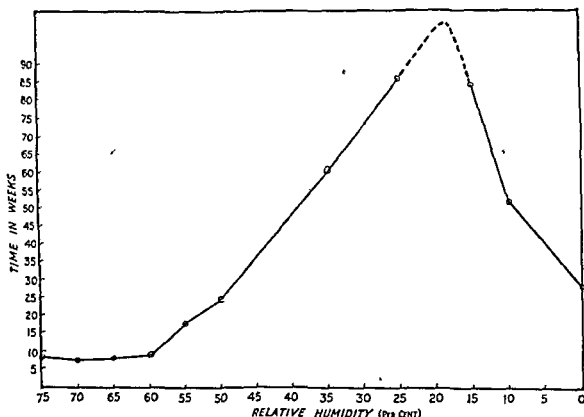


FIG. 3.—Survivor curve showing time taken at different humidities before the numbers of *Str. agalactiae* were reduced by 99.4 per cent.

counts are being maintained, but that of 0 per cent., after remaining at  $1 \times 10^2$  between the 70th and 113th week, fell to nil by the 156th week. It is probable that had observations been made on this part of the curve, the fall would have been shown to be more abrupt. At humidities above 55-60 per cent. there is little difference between the death-rates at different humidities, while at humidities below this zone the differences are considerable.

The actual counts throughout the experiment are given in the table and it will be seen that they are very consistent, the highest throughout being at 25 per cent. relative humidity followed by 15, 10 and 0 per cent. in that order. The only inconsistency is that for a time the count at 35 per cent. was higher than that at 0 per cent.

Since the final time to death is not available for all the humidities, fig. 3 has been constructed to show the time taken to reduce the

number of bacteria from  $150 \times 10^6$  to  $1 \times 10^5$ , *i.e.* for a 99.94 per cent. reduction in number. This shows clearly that the maximum number of viable bacteria is found not at 0 per cent. humidity, as would be expected, but at some point between 15 and 25 per cent. This has held true throughout the experiment.

### Discussion

Only one reference has been found to the effect of the moisture content on the survival of bacteria. Rogers (1914) states that a water content of 5-10 per cent. in slowly dried milk is fatal to a large proportion of cells. In his experiments, however, he was exposing the bacteria to this concentration of water at a temperature of 43° C. in a hot air oven. They had already been kept for two hours at this temperature while the water concentration was reduced from 60 to 10 per cent. and his results are therefore not comparable with those obtained by us during storage at lower temperatures. In the present series of experiments other deleterious factors such as light (Solowey *et al.*, 1942) and oxygen (Elser, Thomas and Steffen, 1935; Siedentopf and Green, 1942) have been constant for all the cultures, the only variable factor being the water content of the atmosphere and hence the moisture content of the substrate by which the bacteria were surrounded. This substrate was colloidal and hence the death-rate was much less than would have occurred had the bacteria been in a non-colloidal medium. The results at the low humidities agree with those of Heller for the duration of his experiments. He dried his cultures in various media and found that during 90 days, *i.e.* 13 weeks, there was little appreciable drop in the number of bacteria when dried in peptone, serum or broth, *i.e.* in colloidal solutions of long chain molecules. On the other hand shorter chain colloids, *e.g.* mucin and tragacanth, gave slow logarithmic death-rates. Steinhaus and Birkeland (1939), in a study of the senescent phase in ageing cultures of *Sarcina lutea* and *Serratia marcescens*, give curves showing a slow decline in numbers during 2 years' storage at room temperature. These approximate to those shown above for the lower humidities. The conditions were very different, since in their experiments the cultures were maintained in nutrient broth, but their results may indicate that in the present series of experiments the bacteria were in the "senescent phase" at the lower humidities and not in a completely "resting phase", as would be expected if all the moisture had been removed. Assuming that *Str. agalactiae* behaves as other streptococci, it will survive longest in the completely dried state produced by vacuum drying of the frozen cultures. If, however, as in these experiments, all the water is not removed then it is possible that the bacteria remain in this "senescent" stage in which they may remain viable longer if there is a certain proportion of moisture in the substrate. The

moisture content of the films was calculated for the various humidities and is given in column 2' of the table expressed as the percentage of the equilibrium weight. From these figures it is seen that at 20 per cent relative humidity the moisture content was approximately 2 per cent. Whether this small proportion of moisture was required for the slight metabolism that may have occurred or was of value in affecting the proteins of the menstruum surrounding the organism is uncertain and no definite explanation can be given. It is possible that Gortner's (1930, 1932, 1938) theory of "bound" and "free" water may partly account for the observed facts.

### Summary

The effect of the relative humidity of the surrounding atmosphere on the survival of *Str. agalactiae* in dried milk films was studied under conditions in which the humidity varied from 0 to 100 per cent. The death rate was not logarithmic at any humidity. The maximal survival rate occurred between 15 and 25 per cent relative humidity and not, as was expected, at 0 per cent. By the method described *Str. agalactiae* can readily be preserved in the living state for three years.

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## ALLOXAN DIABETES IN THE RABBIT

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(PLATES XXIII-XXV)

The work here reported was carried out jointly with the late Professor J. Shaw Dunn and represents his last contribution. Although the paper substantially represents his views, it is felt that it would be contrary to his wish to publish under his name what he had not finally revised.

It has been shown by Shaw Dunn and his collaborators (Dunn, Kirkpatrick, McLetchie and Telfer, 1943a; Dunn, Sheehan and McLetchie, 1943b; Dunn, Duffy, Gilmour, Kirkpatrick and McLetchie, 1944-45) and confirmed by others that the administration of alloxan parenterally to rabbits and other animals causes selective damage to the cells of the islets of Langerhans. The histological evidence of damage may be detected within a few hours after a single considerable dose of the drug. The  $\beta$  cells are the most susceptible and show necrosis under conditions which lead to little or no demonstrable change in the  $\alpha$  cells. The animals, if untreated, manifest acute toxic signs within a few hours of a dose which produces this effect and are likely to die within a day or two. A marked hypoglycaemia is present in the earliest stage and, if the animal survives sufficiently long, this is followed in 1-2 days by hyperglycaemia which persists until death. Below a certain critical dose—about 100 mg./kilo.—no continuing glycosuria was produced and there were no permanent histological changes. Clearly, therefore, it was important to attempt to secure survival over the early period after a large dose in order to investigate the later course of events. It appeared likely that following the initial damage to the islet tissue a condition resembling clinical diabetes mellitus might supervene.

## EXPERIMENTAL OBSERVATIONS

*Induction of the chronic phase*

Initial difficulties in securing survival of the animals were found to depend on two sets of factors. (1) *Varying susceptibility*. It was not recognised at first that individual susceptibility may vary greatly,



so that after a standard dose of the drug, the time of onset and duration of the hypoglycæmic phase cannot be accurately forecast. During this period death tends to occur from hypoglycæmia, with the usual convulsive picture. (2) *The phase of inanition with hyperglycæmia.* In animals which survived the hypoglycæmic stage, a rapid deterioration in the general condition occurred, with anorexia, loss of weight and ketonuria as well as hyperglycæmia and glycosuria. Until a balanced treatment was devised with glucose and, in certain cases, insulin during the first few days after the injection of alloxan, death occurred from inanition. This condition may represent the acute form of clinical diabetes. The following experiments illustrate these points.

**No. 1220** (2.15 kg.). After a fasting period of 24 hours during which unrestricted access to water was allowed, 200 mg./kilo of alloxan (as a 5 per cent. aqueous solution, neutralised) were injected intravenously. Convulsions occurred after 5 hours. Five c.c. of 25 per cent. glucose were then given intravenously with good effect. At 6 hours and 11 hours a dose of 2.5 g./kilo of glucose was given by stomach tube. Next morning this animal was found dead; the blood was not coagulated and its sugar content was estimated at 66 mg. per 100 c.c.

**No. 1207** (2.15 kg.). After feeding on full diet during the preceding 24 hours, 200 mg./kilo of alloxan were given intravenously immediately after a dose of 2.5 g./kilo of glucose by stomach tube. The administration of both was repeated 24 hours later; the same dose of glucose was given again on the 3rd and 4th days. Glycosuria and anorexia appeared on the 3rd day. The blood sugar rose to 533 mg. per 100 c.c. on the 3rd day and to 914 mg. per 100 c.c. on the 4th day. Four doses of glucose, amounting in all to 6 g./kilo, and two doses each of 1 unit of soluble insulin subcutaneously were given on the 5th day. By this time the animal was very thin (weight 1.6 kg.) and lay flat out but with head held up; the eyes were bright and there was slight general tremor; death occurred during the night. *Post mortem* the blood, which was not coagulated, contained over 1 g. of glucose per 100 c.c. The suprarenal cortex showed extensive necrosis.

**No. 1221** (1.8 kg.). This animal was starved for 24 hours but had water to drink; it then received 200 mg./kilo of alloxan intravenously. Six and a half hours later the general condition appeared unaltered, but the temperature had fallen to 100.5° F.; the blood sugar reading at this time was 25 mg. per 100 c.c. There were no signs of convulsions. Glucose (2.5 g./kilo) was given by stomach tube and was repeated next morning. Glycosuria was now present, 11.23 g. of sugar being excreted in the course of the 2nd day. The blood sugar rose to 549 mg. per 100 c.c. on the 3rd day. Thereafter the animal failed to maintain its weight and by the 6th day had lost 450 g. Ketonuria was noted on the 5th and 6th days. On each of the 6th-10th days inclusive about 5 g. of glucose in all were given, either in one or several doses, the oral, intraperitoneal, subcutaneous and intravenous routes all being used, together with 2.5 units of soluble insulin each day subcutaneously. There was a temporary gain in weight until the 10th day, when diarrhoea and lipæmia were observed. The animal became gradually weaker and died on this day.

The histology of the pancreas in these animals is described on p. 207.

In comparison with the above experiments, extended duration of the hypoglycæmic phase is well illustrated by the following animal.

**No. 1211** (2.3 kg.). After normal diet during the previous 24 hours, 200 mg./kilo of alloxan were injected intravenously immediately after

3.75 g./kilo of glucose had been given by stomach tube. The glucose was repeated at 5 hours. On the following morning the animal was convulsed and the blood sugar reading was 30 mg. per 100 c.c. Intravenous glucose (1.5 c.c. of a 25 per cent. solution) was given with good effect. Some minutes later 1.5 g./kilo of glucose was given intraperitoneally. Glycosuria appeared on the 2nd day and persisted until the animal died on the 85th day after excess of insulin.

*Therapeutic measures designed to secure survival beyond the acute stages of hypoglycaemia and of hyperglycaemia with inanition*

*Administration of sufficient glucose to tide over the hypoglycaemic phase.* Usually doses of 2.5-3.75 g./kilo given by stomach tube at about 6, 10 and 24 hours after the injection of 200 mg./kilo of alloxan were effective. Oral administration was preferred to intravenous so as to provide a more constant supply during the hypoglycaemic phase of uncertain duration.

*Treatment of the acute phase of hyperglycaemia and inanition with insulin.* Soluble insulin in total amounts of 1-2 units per day was given subcutaneously, either as one or two daily doses, e.g. in rabbit no. 1193 during the 8th-10th days, and in no. 1224 during the 4th-13th days inclusive. This had the satisfactory effect of stimulating the appetite and leading to increase of body weight. After the first two weeks treatment with insulin was no longer necessary.

*Methods*

Rabbits, mainly of the English type, weighing from 1.6 to 2.5 kg. were used. They were housed in metabolic cages, weighed daily and fed on ordinary diet consisting of 120 g. of a mixture of equal parts of oats and bran moistened with 200 c.c. of water, along with 60 g. of green cabbage leaves; 150 g. of carrot were frequently added to stimulate appetite. Except during experimental periods, unless otherwise specified, the food was given once a day in the morning.

Alloxan as a freshly prepared 2.5 per cent. solution in distilled water was injected intravenously into the marginal vein of the ear; one dose of 200 mg./kilo or two such doses at an interval of 24 hours were the most satisfactory for the present purpose. Such solutions are distinctly acid ( $\text{pH} = \text{ca. } 3$  as determined by dye indicators); but similar lesions were produced by the use of solutions neutralised with dilute ammonia immediately before injection. One animal received only 100 mg./kilo of alloxan intravenously, while another was given 1200 mg./kilo subcutaneously in 10 per cent. solution in a single dose.

Glucose in 25 per cent. aqueous solution was usually given by stomach tube in doses of 2.5-5 g./kilo. Occasionally when convulsions occurred 1.5 c.c. of a 25 per cent. solution were given intravenously.

Urine was collected over 24-hour periods. The volume was measured and the reaction to litmus paper noted: tests were carried out for the presence of albumin (heat), sugar (Benedict's reagents and occasionally the phenyl hydrazine reaction—quantitative estimations by Benedict's method) and ketone bodies (Rother's reaction; a positive Gerhard's reaction was never obtained). A faint haze of albumin was observed in the urine of no. 1193 on the 3rd, 20th and 41st days, and in that of no. 1214 on the 2nd, 3rd and 5th days of experiment. No albuminuria was noted in no. 1215. In five other animals a trace of albumin was found occasionally. In nearly all animals the urinary urea was estimated approximately with sodium hypobromite. No

disturbance of kidney function was noted. Blood sugar estimations were made in duplicate, each with 0.1 c.c. of oxalated blood according to the micro method of Folin and Wu (Harrison, 1937).

*Glucose tolerance tests.* Before the daily feed, 2.5 g./kilo of glucose were given by stomach tube. Blood samples were taken beforehand and at hourly intervals afterwards during a period of 4-6 hours. No food was given on that day until after the last observation.

*Histology.* Constant and even fixation of the pancreas has been difficult to achieve. Most of the common fixatives were tried; Helly's fluid allowed to act for 8 hours gave the best results. Fixed tissues were washed for 2-3 hours, dehydrated in increasing concentrations of ethyl alcohol, cleared in chloroform and embedded in paraffin. Mayer's hæmatoxylin and eosin was used as a routine stain. For cell differentiation and granule staining, Bensley's modification of Mallory's method was employed. Differential counts of the  $\alpha$  and  $\beta$  islet cells were not carried out, but quantitative variations, particularly of the  $\beta$  cells, were so definite that they could be readily assessed. Liver, kidney and psoas muscle were examined for glycogen by Best's carmine method after fixation in alcohol. For the detection of fatty change tissues were fixed in 10 per cent. formalin, and frozen sections made and stained with Sudan IV and hæmalum.

### *Production of persistent glycosuria*

In experiments designed to induce persistent glycosuria, a single intravenous dose of 200 mg./kilo of alloxan was usually employed. As a typical instance, no. 1224 (2.15 kg.) is quoted in full.

Following a period of fasting for 24 hours, but with free access to water, 200 mg./kilo of alloxan in 4 per cent. solution were injected intravenously: 3.75 g./kilo of glucose were given by stomach tube at 4½, 6½ and 11 hours. At 2 hours and 4½ hours blood sugar readings were 202 and 343 mg. per 100 c.c. No signs of hypoglycæmia were observed. Next morning the blood sugar reading was 114 mg. per 100 c.c. Glycosuria was first noted on the 2nd day, when 13.99 g. of sugar were excreted. On the 4th day the animal weighed 1.83 kg.; 5 g. of glucose were given by stomach tube on the 4th and 5th days. From 1 to 8 units of insulin were given subcutaneously each day from the 4th to the 13th, protamine zinc insulin being used in two doses except on the 4th and 5th days, when 1 unit of the soluble preparation was given. Body weight steadily increased from 1.76 kg. on the 5th day to 2.24 kg. on the 13th day. Glycosuria persisted for 272 days, when the animal was killed.

By using a similar procedure, persistent glycosuria was induced in 10 out of 13 animals (table I). Chronic glycosuria was also induced in one animal after subcutaneous administration of alloxan, while 10 per cent. glucose solution was offered it to drink *ad lib.* in addition to its ordinary food (table II). No abnormality in the clinical condition apart from thirst was observed until the 7th day, when glycosuria appeared and persisted with hyperglycæmia until the animal was killed on the 41st day. *Post mortem*, the islets of Langerhans were considerably reduced in number and size. The  $\alpha$  cells appeared normal. The  $\beta$  cells were scanty and those which persisted showed enlargement, degranulation and, more often, vacuolation (fig. 11). No abnormality was noted in the other organs.

The blood sugar reading in all cases was above normal during the phase of glycosuria and usually amounted to between 200 and 400 mg.

TABLE I

*Production of chronic glycosuria in rabbits by alloxan :  
methods and end results*

No (wt in kg)	Dose of alloxan in mg /kilo body weight intravenously	Amount of oral glucose in g /kilo body weight and time of administration in hours relative to dose of alloxan	Day of first appearance (duration of glycosuria in days)	Clinical end result	Islets of Langerhans † ( $\beta$ -cells)
*1193 (1.6)	200, repeated on 2nd day	3.3 at -1, 24, 48	2 (122)	Spontaneous recovery Died 19th day	Normal, hypertrophic, degranulate and hydropic forms
1206 (2.1)	100	3.75 at -1, 6, 24, 30, 48	1 (47)	Diabetic Killed on 47th day	Hypertrophic, degranulate, vacuolated
1211 (2.0)	200	3.75 at -1, 5, 24	2 (83)	Diabetic Died on 85th day after excess of insulin	Hypertrophic, degranulate, very scanty
1214 (2.5)	200	2.5 at 6, 11, 24	3 (162)	Diabetic Killed on 165th day	Hypertrophic, degranulate, vacuolated
1215 (2.0)	100 on 1st day, 200 on 2nd day	3.75 at 24, 48, 72	3 (108)	Diabetic Killed on 201st day	Hypertrophic, degranulate, vacuolated and hydropic forms, very scanty
1219 (2.0)	200	2.5 at 7, 11, 24	6 (20)	Diabetic Killed on 26th day	Hypertrophic, one mitotic figure
1223 (2.25)	200	3.75 at 4, 6, 11	2 (17)	Spontaneous recovery Killed on 197th day	Normal appearance, scanty
1224 (2.15)	200	3.75 at 5, 6, 11	2 (272)	Diabetic Killed on 273rd day	Hypertrophic, degranulate, very scanty
1225 (2.2)	200	3.75 at 4, 6, 11	2 (26)	Spontaneous recovery Redosed on 28th, 35th and 43rd days no definite clinical effect Died on 50th day after tube feed	Normal, pyknotic
1228 (2.25)	200	5 at 4, 6	2 (20)	Sr - - -	degranulate

Nos 1224 and 1228 were starved for 24 hours before administration of alloxan, all the rest were fed normally. Nos 1193 and 1224 received insulin during the early phase of acute hyperglycemia.

\* This animal had received 26, 19 and 17 days previously, three doses of alloxan respectively 25, 100 and 50 mg /kilo, without effect on the sugar metabolism.

† Islets were diminished in number and size, with persistence of well granulated  $\alpha$  cells and sometimes enlargement, except in nos 1193 and 1225 in which the islets were normal in size and number.

TABLE II

*Rabbit no. 1247 (2.6 kg.). Details of treatment*

	Alloxan (mg /kg) as a 5 or 10 per cent aqueous solution	Glucose per os in g /24 hrs
1st day	300	15.5
2nd "	600	22.5
3rd "	0	15.6
4th "	1200	22.5
5th "	0	9.5
6th "	1200	1.0
7th "	0	17.8

per 100 cc. Spontaneous recovery occurred in four of the rabbits (nos 1193, 1223, 1225 and 1228) after 122, 17, 26 and 20 days respectively.

*Clinical observations*

During the first two weeks most animals showed some loss of weight—up to 10 per cent.—but this was usually regained on ordinary feeding, with or without the addition of 150 g. of carrot per day, or after the administration of 1-2 units of soluble insulin daily, with or without oral glucose. Weight thereafter was generally maintained at the pre-experimental level, but definite increase beyond this point seldom occurred. The addition of carrot to the diet was followed by an increase in the general food intake and by raised blood sugar, urinary volume and amount of sugar excreted. Similar effects were obtained on giving a double daily feed over a period of one week. Withdrawal of carrots and greens had the opposite effect of diminishing the intake of other food and lessening urinary volume and sugar. The effect on the blood sugar was less definite. The urine became acid and there was usually some loss of weight. Withdrawal of all food, apart from water, for 24-48 hours caused both temporary return of the blood sugar to normal and disappearance of sugar from the urine. Definite polyuria was observed in nos. 1193, 1214, 1215 and 1224, as much as 500 c.c. of urine and 40 g. of sugar being excreted during 24 hours. Ketonuria was not present except during the first few days in animals which died acutely and at the chronic stage after special feeding (see below).

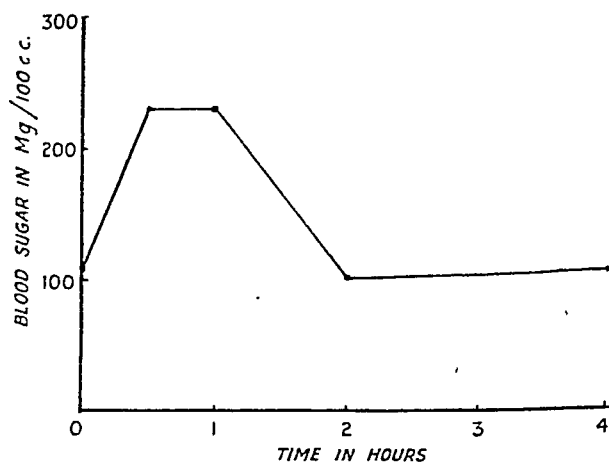


FIG. 1.—Sugar tolerance curve from normal rabbit, after 2.5 g./kg. oral glucose (glycosuria absent).

*Cataract.* The development of cataract in both eyes was observed in nos. 1193, 1214, 1215 and 1224. The last was regularly examined over a period of four months. According to the report of Dr A. Loewenstein who made the investigation in the Tennent Institute of Ophthalmology, changes were mainly referable to the lens and resembled closely those characteristic of human diabetic cataract. Retinal changes such as occur in diabetes in man were absent; this may be explained by the relatively short duration of the condition in rabbits.

*Effect of insulin.* The subcutaneous administration of suitable doses of protamine zinc insulin was followed by a return of the blood sugar to normal and aglycosuria within 24 hours. It was found that the daily doses required to produce this effect, which persisted only so long as insulin was continued, were respectively 10, 16, 25 and 15 units in nos. 1193, 1211, 1215 and 1224.

*Glucose tolerance tests.* Definite evidence of diminished tolerance of glucose at various stages of the condition was given by nos. 1193, 1214, 1215 and 1225 (figs. 1-3). In animals in which spontaneous recovery occurred, the curves

afterwards showed some delay in the return of the blood sugar to normal; no glycosuria was noted.

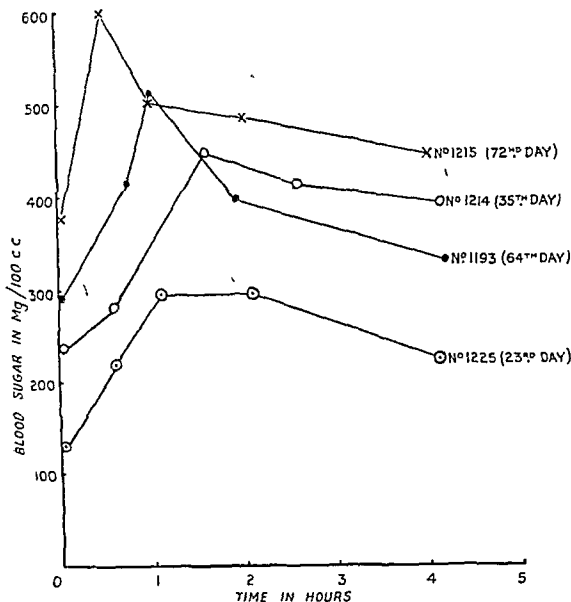


FIG. 2.—Sugar tolerance test—diabetic curves (glycosuria present).

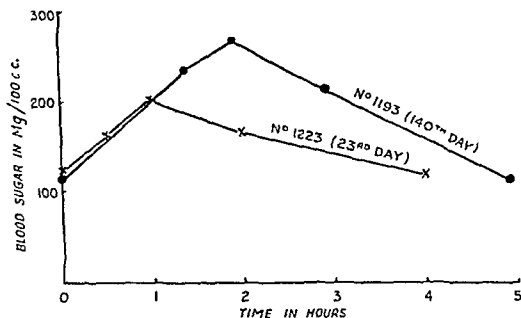


FIG. 3.—Sugar tolerance curves after spontaneous recovery (glycosuria absent).

*Effects of special diets.* With a view to producing ketosis, soya bean, olive and nut oil and glucose were added to the diet of certain animals, as follows.

No. 1206. About 80 g. of soya bean meal instead of oats were given, with 60 g. of bran and 150 g. of carrot, from the 16th to the 27th day inclusive. One-half to two-thirds of the mixture was eaten daily. There was no increase in the output of urine or urinary sugar, the average output of which was 3.23 g. per day during this period. The blood sugar readings were 266, 445 and 302 mg. per 100 c.c. on the 16th, 22nd and 26th days respectively, compared with 410 and 327 mg. per 100 c.c. on the 5th and 12th days. Body weight increased from 2040 to 2270 g. Ketonuria was observed on the 21st day only.

No. 1193. On the 57th day the blood sugar was estimated at 142 mg. per 100 c.c. and 19.1 g. of sugar were excreted on the 58th day. Ten c.c. of olive oil were added to the ordinary feed, all of which was consumed, on the 60th and 61st days. The blood sugar on the 61st day was 124 mg. per 100 c.c. and the urine was sugar-free on the 60th, 61st and 62nd days. - There was no ketonuria.

No. 1214. Olive oil and nut oil were given from the 11th to the 18th days, 20 c.c. of one or the other being added daily to the usual feed of oats, bran and carrot, which was taken well. The weight increased from 2510 to 2640 g. There was no diminution in the blood sugar, which was estimated at 471 mg. per 100 c.c. on the 15th day, or in the urinary sugar. The animal looked well and an oily, soft texture was imparted to the skin and fur. Ketonuria did not occur.

No. 1224. Between the 53rd and 63rd days, 10 per cent. glucose solution was offered to the animal in addition to the usual quantity of oats and bran. An appetite for glucose was shown, 48.67 g. on the average being taken per day in addition to the usual intake of ordinary food. The blood sugar rose from 309 mg. per 100 c.c. on the 49th day and fluctuated between 421 and 654 mg. per 100 c.c., and a 10 per cent. increase in weight occurred. An average of 516 c.c. of urine and 34.8 g. of sugar were excreted per day. Ketonuria was distinct between the 56th and 66th days.

### *Morbid changes \**

On post-mortem examination, no obvious naked eye lesions were visible in any organ except in expt. 1193; this animal died of intestinal obstruction after six months, induced possibly by a period of enforced reduction of intake of water and greens. The aorta showed irregular dilatation, roughening of the lining from the valve to the bifurcation and the presence of yellowish patches, as sometimes met with in apparently healthy animals. Fatty change in the intima was confirmed microscopically. In all animals the kidneys, liver, thyroids, parathyroids and suprarenal glands were examined histologically. A tubular lesion was found in the kidneys of only three animals, all of which showed prolonged glycosuria. In two of these (nos. 1214 and 1215) there was involvement of a small proportion of the 2nd convoluted tubules, the epithelium being necrosed and replaced by a structureless, hyaline material with an affinity for hæmalum, indicative of early calcification. The kidney of no. 1193 was more extensively affected, showing considerable atrophy of the 2nd convoluted tubules and a conspicuous increase of intertubular connective tissue. Foci of lymphocytic infiltration were noted in

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\* Grateful acknowledgment is made to Professor D. F. Cappell for critically reviewing the histological results.

## ALLOXAN DIABETES IN THE RABBIT

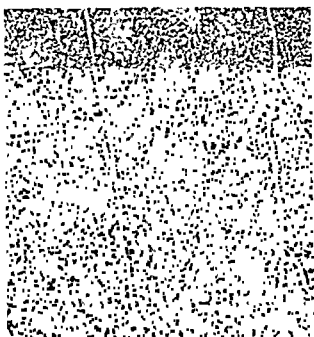


FIG. 4.—Normal rabbit pancreas showing frequency of distribution and size of the islets of Langerhans. H. and E.  $\times 60$ .

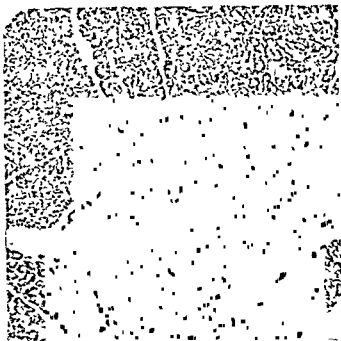


FIG. 5.—Rabbit no. 1211. Section of pancreas containing a few tiny islets which are inconspicuous at this magnification. H. and E.  $\times 60$ .

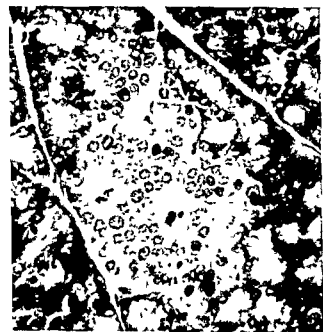


FIG. 6.—Normal islet of Langerhans from rabbit. H. and E.  $\times 350$ .



FIG. 7.—Rabbit no. 1206. Small islet from diabetic animal showing hypertrophic  $\beta$  cells with diminished granules. Bensley-Mallory.  $\times 600$ .





many places around damaged tubules. In all these animals the glomeruli and vessels appeared normal. These lesions no doubt represent the late effects of acute damage to the kidney which Shaw Dunn and his collaborators originally described as occurring in a proportion of animals after a considerable dose of alloxan, the first and sometimes the second convoluted tubules being the elements especially affected. It is possible that the chronic kidney lesion may have contributed to the death of rabbit 1193; this animal had received exceptional treatment in that it had previously been given a series of small doses of alloxan without effect on its sugar metabolism. It had been noted, however, that intermittent albuminuria had resulted. It must be emphasised that this intercurrent nephritic condition had no essential relation to the production of the chronic glycosuria, since the majority of the diabetic animals showed no renal damage. This is particularly striking in the case of no. 1224, which developed the most severe and prolonged diabetic state, yet there was no kidney lesion. The suprarenal cortex presented lesions in only three animals, which died within 24 hours (no. 1220), 7 days (no. 1207) and 10 days (no. 1221). The damage was most severe in no. 1207, which showed necrosis in all layers except the zona glomerulosa, where a few mitoses were seen. In nos. 1220 and 1221 there was only some shrinkage of the cytoplasm and pyknosis of the nuclei in a small number of the cells in the zona fasciculata and zona reticularis. As mentioned later, these animals presented a picture comparable with that of acute human diabetes. Since the question arises in how far the suprarenal cortical lesions may have aggravated the condition, it is noteworthy that there was a marked lesion in only one of the three. No definite changes were observed in the other endocrine organs. In the animal showing the most severe condition and longest duration (no. 1224), the pituitary body was examined with negative results. In all the animals glycogen was present only in small amount or was entirely absent from liver and muscle. In the kidney, the positive correlation between the presence of glycogen in the tubular epithelium and the existence of glycosuria as noted by Warren (1938) and others was confirmed. Fatty change in the liver was seen in only one case (no. 1214), when it was present in small amount as fine droplets evenly distributed throughout the lobules. To the naked eye the pancreas did not differ from normal, but striking lesions were seen microscopically.

#### *Histology of the pancreas*

*Early stage (1-10 days).* In no. 1220, which died within 24 hours, the changes in the islets of Langerhans were those of advanced necrosis as already described by Dunn *et al.* (1943 *a* and *b*). In nos. 1207 and 1221, which died on the 6th and 10th days respectively, there was very great diminution in the amount of islet tissue, which

was now composed mainly of  $\alpha$  cells lying in close apposition with each other and with the acinar tissue. They showed a healthy appearance, with a distinct cell outline. Very few  $\beta$  cells were seen. Some of these appeared normal, others showed loss of cytoplasm and granules. In no. 1207 there was partial atrophy of the central acinar tissue.

*Late stage* (20-272 days). The islets were always reduced in number and size (*cf.* figs. 4 and 5), except in nos. 1193 and 1225. The outline of the islets was often irregular and the general architecture was frequently disturbed, the usual ribbon arrangement not being present (*cf.* figs. 6 and 10). Thus the persisting islets appeared as clumps of cells without interspersed capillaries. It is left open as to whether this appearance indicates multiplication of surviving islet cells leading to a greater or less degree of regeneration of the islet, or whether it results from collapse of the persisting cells after disappearance of necrosed elements. The proportion of  $\alpha$  and  $\beta$  cells was usually equal instead of being about 1 to 10-15 as normally. No definite statement can be made regarding any sequence of changes in the islets in the period intermediate between early and late stages.

The  $\alpha$  cells were sometimes arranged marginally or occupied half the islet (figs. 9 and 10); in some cases they were the only constituent cells. They were often enlarged, the nucleus appearing healthy and the cytoplasm closely packed with fuchsinophile granules, while the cell outline was well defined. A few single  $\alpha$  cells were seen in the acinar tissue, and although there was no evidence to suggest that they were vestiges of former islets, this cannot be excluded, since  $\beta$  cells may disappear without leaving any trace.

The  $\beta$  cells, which were reduced in number throughout the series—in some cases very markedly—showed considerable variation in structural appearance even in adjacent islets. Many were normal, others were increased in size, well granulated and contained large vesicular nuclei. In a few the nuclei were pyknotic, while the outline of the cell, although indistinct, did not appear shrunken. In seven of the surviving animals the cytoplasm was depleted of specific granules in varying degree (figs. 7 and 8), and in three of these the completely degranulated cells contained a fairly coarse “reticulum” with interstitial vacuolation (fig. 11). Without a more specific stain for  $\beta$  cells it is not possible to state whether this appearance represents true cytoplasmic vacuolation or whether the reticular framework is a normal feature which is rendered visible on the disappearance of the specific granules. A mitotic figure was seen in a  $\beta$  cell in no. 1219; this is a most uncommon finding. Hyalinisation, lymphocytic infiltration and interstitial fibrosis were not seen. The epithelium of the small ducts showed no variation from normal.

The following experiments are quoted on account of the special changes found.

## ALLOXAN DIABETIS IN THE RABBIT



FIG. 8.—Rabbit no. 1206 Islet showing two deeply staining  $\alpha$  cells and numerous hypertrophied  $\beta$  cells depleted of their specific granules. Bensley-Mallory.  $\times 600$ .



FIG. 9.—Rabbit no. 1206 Islet showing preponderance of well granulated  $\alpha$  cells. The capillaries are also deeply stained. Bensley-Mallory.  $\times 600$ .

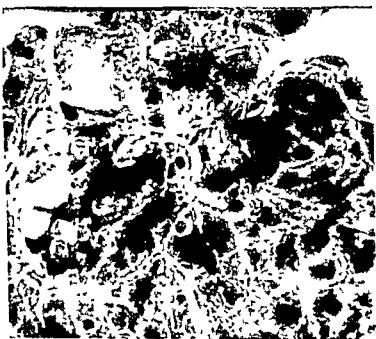


FIG. 10.—Rabbit no. 1211 Deformed (bilobed) islet from diabetic animal consisting mainly of deeply staining  $\alpha$  cells, with a group of hypertrophied and degranulated  $\beta$  cells towards the left-hand margin. Bensley-Mallory.  $\times 600$ .



FIG. 11.—Rabbit no. 1247. Islet from diabetic animal showing vacuolation and a fairly coarse 'reticulum' within the  $\beta$  cells. Bensley-Mallory.  $\times 600$ .



No 1193. This animal, which died on the 194th day, became aglycosuric and apparently returned to normal after 122 days. The number and size of islets were within normal limits. Their outline, however, was often irregular.  $\alpha$  cells were present in increased proportion and appeared healthy, without evidence of enlargement. The  $\beta$  cells were reduced in number, some were normal in appearance, but the majority showed hypertrophy and depletion of granules. The nuclei in many were shrunken and hyperchromatic, while the cell bodies were considerably swollen, with a clear cytoplasm almost devoid of stainable granules. This change was recognised as hydropic degeneration (fig. 13).

No. 1215. This animal showed glycosuria from the 3rd day until it was killed on the 201st day. There was considerable reduction in the number and size of the islets, which were composed mainly of  $\alpha$  cells. The latter were slightly enlarged, with healthy nuclei and abundant fuchsinophile granules in the cytoplasm (fig. 12). The  $\beta$  cells, which were considerably diminished in number, were enlarged and contained large vesicular nuclei. In the majority, the cytoplasm showed complete or almost complete degranulation but in many of the others there was the appearance of vacuolation. A further small number showed the marked distension of the cell body, clear cytoplasm and sometimes nuclear pyknosis indicative of hydropic degeneration (fig. 12).

No 1206. Persistent glycosuria was present until the 47th day, when the experiment was terminated. In addition to the features already described, a small proportion of the persisting islets were considerably enlarged, consisting of hypertrophic  $\beta$  cells (fig. 14).

In No. 1211 a section stained with hæmalum and eosin showed one lobule of exocrine tissue pale in colour, with a collapsed shrunken appearance (fig. 15). The cells were large and vesicular, with poor affinity for hæmalum and diminished content of eosinophile material. This was the only case, apart from no 1207, where any exocrine abnormality was observed.

## DISCUSSION AND CONCLUSIONS

The immediate effect of a considerable dose of alloxan (200 mg /kilo) administered intravenously may conform to one of two types, in either of which death may occur if the animal is untreated. (1) There may be prolonged and profound hypoglycæmia which develops within several hours and is characterised by the usual convulsive phenomena. This is probably associated with the liberation of a large amount of insulin from  $\beta$  cells of the islets rendered necrotic by the drug. This view has been expressed by Hughes *et al* (1944) and has been supported by recent work carried out by Ridout *et al* (1944). In this form death occurs usually within 24 hours. (2) In animals in which the above phase is less prolonged or less severe there follows an acute diabetic condition, explicable on the basis of a rapid exhaustion of the insulin reserve, which presumably causes depletion of the glycogen content of the liver. There is then marked hyperglycæmia, glycosuria, anorexia, loss of weight and sometimes ketosis, and death occurs from inanition in a week or more. Administration of glucose in these circumstances tends to aggravate the clinical state, leading to ketonuria and lipæmia. It has been shown in previous publications that the condition underlying these changes is an acute necrosis of the islets of the pancreas, which affects almost exclusively the  $\beta$  cells. The clinical picture in the latter type may be analogous to acute diabetes in man.

Survival beyond these stages of acute damage may be secured by administration of sufficient glucose and insulin during the hypoglycæmic and hyperglycæmic phase respectively. In those treated animals which survived the acute stages the effect of alloxan was to produce a condition which closely resembles chronic human diabetes. The clinical features in the rabbit are hyperglycæmia, glycosuria, polyuria and polyphagia, also delayed clearance of sugar from the blood after administration of glucose to the fasting animal. Further, the condition is "insulin sensitive" as shown by the rapid fall in the blood sugar and glycosuria after administration of insulin. Much more experimental work is needed to determine whether the glucose tolerance and the amount of insulin required to adjust the sugar metabolism to normal limits are directly related to the condition of the islets as observed after death. A comparison of nos. 1193 and 1215 suggests that when there is extensive reduction of islet tissue, as in the latter animal, a more severe "diabetic curve" is manifested and a greater dosage of insulin is required than when the loss of islet tissue is slighter, as in no. 1193.

There are certain other features which seem to be characteristic of the chronic disease produced by alloxan in the rabbit, namely absence of ketosis on ordinary diet and capacity for spontaneous recovery in a proportion of cases. In experiments designed to produce ketosis, diets were given containing respectively a preponderance of fat (nut oil and olive oil), protein (soya bean) or carbohydrate (glucose). It was found that on a high carbohydrate diet the excretion of ketone bodies in the urine was most pronounced, although insufficient for detection by Gerhardt's test, while in addition hyperlipæmia became very marked. Transient ketonuria also followed the addition of protein. On a diet containing olive oil and nut oil no ketonuria and no lipæmia occurred; there was improvement in the general condition, although slight reduction of hyperglycæmia and glycosuria was found in only one of two animals. These observations recall the work of Marks and Young (1939) with pituitary-diabetic dogs. They found that ketonuria was maximal on a meat diet and lowest with prolonged fat diet. For these and other reasons they suggested that the excretion of ketone bodies is particularly associated with the oxidation of muscle protein. Although the conditions determining ketosis in the rabbit require further examination, it appears that interference with the oxidation of ketone bodies occurs when the islet cells have been temporarily exhausted of insulin owing to an excess of carbohydrate (particularly glucose) in the diet, and, in view of the hyperlipæmia, probably when the glycogen of the liver and muscles has been considerably depleted. Accordingly, it may be that the absence of ketosis in the diabetic rabbits on ordinary diet is dependent upon its low protein content and the presence of a sufficient amount of glycogen in the tissues.

The fact of spontaneous recovery implies, of course, that a per-

## ALLOXAN DIABETIS IN THE RABBIT

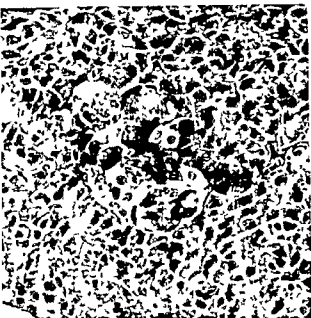


FIG. 12—Rabbit no. 1215—diabetic. Small islet of Langerhans showing slightly enlarged  $\alpha$  cells (more deeply staining) and pale, degranulated and hydropic  $\beta$  cells. Bensley-Mallory. (Panchromatic plate; Wratten B. and G. filters)  $\times 300$ .

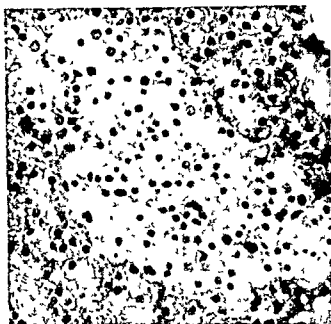


FIG. 13—Rabbit no. 1193. There is hydropic degeneration of the  $\beta$  cells with hyperchromasia and nuclear pyknosis. H. and E.  $\times 300$ .



FIG. 14—Rabbit no. 1206. Section of the pancreas showing a hypertrophic islet composed mainly of  $\beta$  cells. H. and E.  $\times 175$ .

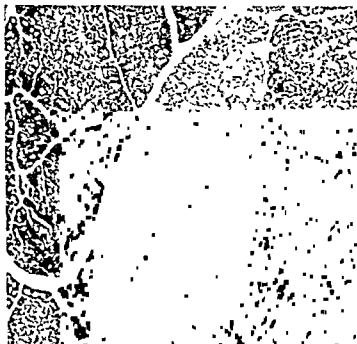


FIG. 15—Rabbit no. 1211. Section of the pancreas showing loss of staining affinity in one lobule of exocrine tissue. H. and E.  $\times 60$ .





manently increased supply of insulin has become available for readjustment of metabolism. This might be explained in one of two ways, either by the persistence of a sufficient reserve of  $\beta$  cells which had recovered from damage short of necrosis, or by a certain proportion of  $\beta$  cells escaping damage altogether and then undergoing hypertrophy or hyperplasia or both. Although Woerner (1938) has recorded hyperplasia of the islet cells following prolonged intravenous administration of glucose, the conditions of his experiments were different from those of the present series. Actually, owing to the rarity of mitoses in the persisting islet cells, definite indications of hyperplasia are lacking. On the other hand, histological changes indicative of hypertrophy and hyperfunction of surviving cells, *i.e.* enlargement and depletion of specific granules, have been observed in all the diabetic animals, including one of the four which recovered spontaneously and lived free from glycosuria for a period of over two months. Spontaneous recovery may, however, be associated with great reduction in the number of islets (no. 1223), but without hypertrophy and degranulation of the  $\beta$  cells. Accordingly it must be concluded that sometimes a small fraction of persisting islet cells can carry out the endocrine function sufficiently for the metabolic needs of the animal.

Although hypertrophy of the  $\beta$  cells and depletion of specific granules were constant findings associated with a diminished tolerance for glucose, certain of the animals were capable of utilising a part of the carbohydrate in their diet, of maintaining weight and of spontaneous recovery. In view of this and also of the considerable diminution in number of  $\beta$  cells, it is suggested that these histological changes in the  $\beta$  cells are indicative of overactivity under the stimulus of a high blood sugar content and that this may be an exaggeration of the normal mode of functioning. The results of two experiments (nos. 1193 and 1215) suggest that prolonged hyperfunction may be followed by hydropic change.

Persistence of healthy and often enlarged  $\alpha$  cells was a striking feature of all the sections examined, which points to a distinct difference in form and function as compared with  $\beta$  cells. A similar persistence of  $\alpha$  cells has been recorded in pituitary diabetes in dogs by Richardson (1939-40) and in dogs after the administration of alloxan by Goldner and Gomori (1943). These cells do not appear to take part in the production of insulin, but no evidence was obtained to indicate what their function may be. No definite changes were present in any other organs to suggest that they played an essential part in the aetiology of the clinical condition.

It is concluded that the cardinal signs of diabetes mellitus can be reproduced experimentally in rabbits which have survived the injection of a pure chemical substance causing specific damage to the  $\beta$  cells of the islets of Langerhans.

## SUMMARY

1. A large intravenous dose of alloxan (200 mg./kilo) causes rapid death in rabbits, there being a phase of hypoglycæmia, followed by hyperglycæmia and inanition if the animal survives for several days. *Post mortem* the chief change is damage to the cells of the islets of Langerhans, the  $\beta$  cells being especially affected. Survival beyond this acute toxic phase may be secured by appropriate treatment with glucose *per os* during the first 48 hours, followed sometimes by insulin during the first two weeks.

2. Animals which survive the acute toxic phase develop a condition similar to human diabetes, with hyperglycæmia, glycosuria, polyuria, polyphagia and cataract. There is diminished tolerance of glucose, but tolerance is increased by administration of insulin. Ketonuria, except at the early phase, is not a feature on ordinary diet but may be induced by administration of excess of glucose.

3. In a proportion of cases spontaneous recovery occurs after several weeks or months, but animals have remained diabetic for as long as 6-9 months.

4. The only constant lesion in the diabetic rabbits is diminution in size of the pancreatic islets and reduction in number of  $\beta$  cells, which also show enlargement, degranulation and sometimes vacuolation and hydropic change. The  $\alpha$  cells are practically unaffected.

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# FATAL BRONCHIAL ASTHMA SHOWING THE ASTHMATIC REACTION IN AN OVARIAN TERATOMA

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(PLATES XXVI-XXVIII)

DEATH in an attack of bronchial asthma is unusual, and reports of cases proved by the exclusion of an extra-thoracic cause of death at autopsy are rare. There is no agreement as to the number of cases in the literature; some are rejected as having had asthma but not having died during an attack and others are not regarded as being examples of true bronchial asthma. For example, Michael and Rowe (1934-35) considered that only 29 of the 49 reported cases of bronchial asthma coming to post-mortem were true examples of this condition. As in some of the less striking rarities, the number of published cases gives an exaggerated picture of their infrequency, and a better indication of the incidence is given by Thieme and Sheldon (1937-38). Out of 2060 autopsies at the Michigan University Hospital between July 1932 and April 1937 they found 7 examples of death in an attack of bronchial asthma. Similarly Craig (1941) found 7 such cases among the 23 asthmatics submitted to post-mortem examination in 56,000 admissions to the Peter Bent Brigham Hospital. Including his own, he estimated the number of genuine cases to that date as 59.

The association of death in an asthmatic attack with typical asthmatic changes in an ovarian teratoma would seem to be unique.

## Case report

*Clinical history.* A female aged 47 had suffered from bronchial asthma "for years". She was admitted to the Royal Victoria Infirmary, Newcastle-on-Tyne, under Dr A. G. Ogilvie on 15.4.44. On admission she was wheezing a little and at 11 p.m. on the same day she had a typical asthmatic attack relieved by adrenalin. No abnormal signs were noted in heart or lungs apart from rhonchi and prolonged respirations. At 1 a.m. on the following morning she was restless and was given  $\frac{1}{4}$  gr. of morphine. At 8.30 a.m. she was found sitting upright with her head hanging down. She was collapsed and cold, with cyanosis of the lips. Respirations were shallow and grumbling. She was given 1 c.c. of coramine but died half-an-hour later. Autopsy was performed 48 hours after death.

*Post-mortem findings*

*Anatomical diagnosis.* Pulmonary emphysema with excess of mucus in bronchi. Persistent thymus. Dermoid cyst of ovary. Cholelithiasis with gall-stones in common bile duct. Multiple uterine myomata. Adenoma of thyroid. Polypus of sigmoid colon. Obsolete tuberculosis of mesenteric lymph nodes.

The significant findings were as follows. Heart 320 g., valves healthy, both ventricles dilated (right 0.4 cm. thick, left 1.8 cm. at base). Spleen 78 g., pulp firm and featureless. Liver 1210 g., congested. Kidneys 255 g., no abnormal appearances. Thymus 12 g. Gall-bladder contained 8 mixed faceted gall-stones 1.2 cm. in diameter. Common bile duct slightly dilated and contained 4 faceted gall stones 0.8-1.0 cm., lying free. Uterus enlarged and contained 5 subserous fibromyomata 1.4 cm. in diameter. Pleural sacs—left, no excess of fluid; right, largely obliterated by old adhesions. Lungs—left, 450 g.; right, 520 g.: both were pale, swollen and emphysematous, filling the pleural sacs and meeting in the mid-line. There were scattered petechial hæmorrhages in the pleuræ over the left lower lobe posteriorly and right lower lobe laterally. The cut surfaces were uniformly emphysematous, without bullæ. There was some congestion posteriorly but the lungs were elsewhere dry and pale and cedema was absent except for a little at the base of the right lower lobe. The bronchi were thick-walled and prominent, projecting from the cut surface, and the most striking feature was the tenaceous jelly-like mucus in their lumina. The main bronchi at the bifurcation contained only strands of this ropy mucus, but all others of 1 cm. diameter down to the smallest recognisable with the naked eye were more or less completely filled. This was especially evident in the portions of lungs fixed in formalin for microscopical examination, when the coagulated mucus formed translucent casts. The bronchial mucosa was everywhere deeply congested. Left ovary normal. The right ovary was replaced by a dermoid cyst  $12 \times 11 \times 9$  cm. in diameter, filled with yellowish opaque fluid containing tangles of black hair and solid masses of sebaceous material. The teratomatous nodule in the wall of the cyst measured  $2 \times 1.5 \times 1.5$  cm. and from it projected a tuft of coarse black hair. No bones nor teeth were present and yellow adipose tissue was the only tissue recognisable on section. The other organs showed no significant changes. Brain not examined.

*Microscopical appearances*

*Lungs.* These show with unusual clarity the changes which occur in death from bronchial asthma, as the picture is not confused with secondary changes such as infection or cedema. All the bronchi with cartilage and glands are more or less filled with mucus; some are apparently completely occluded (fig. 1), while others show a central cavity of variable diameter. The mucus has a fibrillary appearance and is almost unstained in hæmalum and eosin sections, but it stains brightly with mucicarmine. Its cellular content is low and very uniform—desquamated ciliated columnar epithelial cells of the bronchial mucosa and eosinophils in roughly equal proportions, with less frequent macrophages and scanty polymorphs. The desquamated epithelial cells are not confined to the mucus at the margin; some are present in the interior of the mucous plug. The lining epithelium

## ASTHMATIC REACTION IN AN OVARIAN TERATOMA



FIG. 1—Small bronchus showing marked contraction and thickening of the wall, with a plug of mucus almost occluding the lumen H and L  $\times 10$

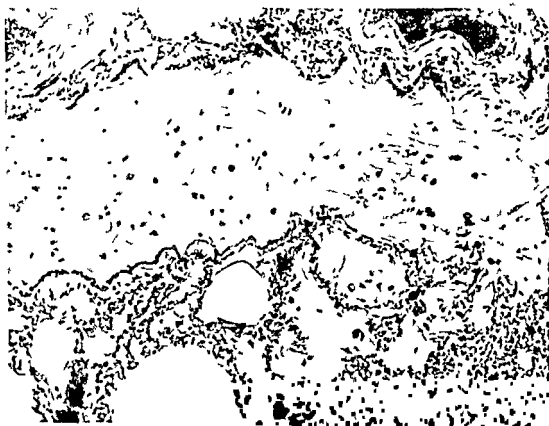


FIG. 2—A terminal bronchiole filled with mucus which extends into the adjacent air vesicles H and E  $\times 150$



still *in situ* is represented mainly by the deeper cells, but where the surface layer survives goblet cells appear to be more frequent than usual. The contracted state of the bronchi is evident in their accentuated wrinkling and crenation. The mucosal basement membranes are much thickened, hyaline and structureless. The mucosa is appreciably broadened and shows a dense cellular infiltration, the great majority of the cells being mature two-lobed eosinophils (fig. 6). This eosinophilic infiltration is not sharply confined to the mucosa; it extends into the submucosa and adjacent septa and the peri-bronchial tissues generally. Eosinophils are scanty in the bronchial glands and muscle, but abundant in the connective tissues between them. There are scattered lymphocytes as well as small lymph follicles in the submucosa. The bronchi are congested, with frequent dilated blood vessels in the submucosa; a few dilated capillaries are packed with solid masses of eosinophils. The bronchial glands are hypertrophied and prominent, and their ducts, which are dilated and filled with mucus, show hyaline thickening of the basement membranes. The bronchial muscle generally is hypertrophied. These changes are present in all the larger and medium sized bronchi. In the smaller bronchi without mucous glands or cartilage and in the bronchioles the appearances are much more variable. About half show changes similar to those in the larger bronchi, differing only in degree. The mucus, which generally fills them, contains few cells other than macrophages, and in particular the ciliated columnar epithelial lining has not desquamated and consists mainly of goblet cells. The basement membranes are thickened and hyaline and the muscle appears hypertrophied, but the mucosal eosinophilic infiltration is less marked. The remaining bronchioles, consisting of the majority of the terminal ones, contain no mucus and are collapsed, with irregular distorted lumina. The lining cells are detached in sheets and goblet cells are scanty or absent. Basement membranes are not thickened and eosinophils are practically absent. A few however of the terminal bronchioles are distended with mucus, and not infrequently this extends into the associated air vesicles (fig. 2). This filling of the air vesicles with mucus is not associated with any cellular or other local reaction.

With methylene blue and eosin, Charcot-Leyden crystals stand out prominently as red in the practically unstained mucus in the lumen of the bronchi. They occur singly and in clusters and vary greatly in size; a few are small and some of these are inside the cytoplasm of macrophages, but the majority are larger. In the bronchial mucosa, at the sites of maximum eosinophilic infiltration, Charcot-Leyden crystals are common but here all are small, none being longer than twice the diameter of an eosinophil. They are not present in the bronchial mucous glands or their ducts, and presumably these small crystals pass through the thickened basement membranes to the lumen of the bronchus and there grow by accretion of the



products of eosinophilic disintegration. No Charcot-Leyden crystals are evident inside the cytoplasm of the eosinophils. In the smaller bronchi a few Curschmann's spirals are also present (fig. 3). The only vascular changes noted are occasional thrombi in small pulmonary arterioles, all of them recent and only one showing early organisation. No lesions are seen in the vessel walls and there is no eosinophilic infiltration.

*Liver.* This shows the changes of early chronic venous congestion. The cells in the central and intermediate zones are shrunken and degenerating and their nuclei are pyknotic.

*Thymus.* This consists of vascular and oedematous adipose tissue, with islands of lymphoid tissue containing scanty Hassall's corpuscles.

*Kidneys.* Glomeruli are large and capsular spaces are reduced or apparently obliterated. There is slight granularity and swelling of convoluted tubular epithelial cells.

*Myocardium.* This shows no obvious abnormality.

Eosinophils are not evident in any of these four organs.

*Dermoid cyst of ovary.* The only material available for study is a paraffin block of one-half of the mural teratomatous nodule. It is covered at one end by keratinising squamous epithelium (fig. 4) resembling skin and with abundant sebaceous and sweat glands and a few strands of unstriated muscle in the underlying connective tissue. This changes into non-keratinising squamous epithelium for a short length, which in turn is replaced by pseudo-stratified columnar epithelium of respiratory type. From the surface two narrow clefts extend for a short distance into the interior, one covered by similar epithelium, the other by a single layer of ciliated columnar cells. As in the bronchi, the surface ciliated epithelium has largely desquamated but it survives in small areas. Beneath this respiratory type of epithelium is connective tissue containing much unstriated muscle and deep to this are frequent mucous glands. The respiratory epithelium on the surface of the nodule gradually thins to an occasional isolated epithelial cell at the other end of the section, where mucous glands are lacking. No cartilage is present and there is nothing resembling lung tissue. Deep to the tissues described are small foci of dilated sweat glands, adipose tissue, small bundles of peripheral nerve fibres and a tube of unstriated muscle lined by mucosa of intestinal type.

The resemblance of the epithelium termed respiratory to that of the bronchi is heightened by the presence of similar "asthmatic" changes in the underlying tissue. The basement membrane is much thickened and hyalinised, and under it is a dense focal infiltration of eosinophil cells (fig. 7). This has a very precise distribution, none being present under the skin and only a few under the short length of non-keratinising squamous epithelium. It extends to the connective tissue between the bundles of unstriated muscle and between the mucous glands, but not to that around the muscle bundles, sebaceous

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FIG. 3—Curschmann spirals in small bronchus. Methylene blue and eosin  $\times 120$

FIG. 4—Ovarian teratoma. Section from the thin covered portion of the teratomatous capsule showing a serous gland but no evidence of infiltration by eosinophil cells. H and E.  $\times 100$



FIG. 5—Ovarian teratoma showing eosinophil cell infiltration of respiratory type mucosa with frequent small Charcot-Leyden crystals. Methylene blue and eosin  $\times 1200$

it as mucus and do not mention the use of mucicarmin stain. Its presence in the air vesicles is presumably the result of inspiratory efforts when the bronchi are largely occluded by mucus. In another recent case of death in an asthmatic attack I was able to demonstrate a similar amount of intra-alveolar mucus.

Clinically these cases have an asphyxial death from bronchial obstruction, and there has been much discussion as to the relative importance of the three generally accepted factors—over-production and retention of mucus, muscular contraction of bronchi, and mucosal œdema. It would appear that these have not the same significance in fatal and non-fatal asthmatic attacks. In the latter the obstruction is relieved by adrenalin, and it is reasonable to suppose that the contraction of the bronchial muscles is the major factor in determining the obstruction. The majority of the cases with which this paper is concerned are examples of status asthmaticus, in which adrenalin and similar drugs are ineffective in relieving the condition. They are generally described as adrenalin-fast, implying that adrenalin has failed to produce a relaxation of the bronchial muscles. But it is doubtful if this is a justifiable assumption, as the main cause of bronchial obstruction found in the majority of these cases at autopsy is blockage by mucus. The contraction of the bronchial muscle is presumably relieved by adrenalin in these cases, as in those with non-fatal attacks, but the blockage by mucus persists, and clinically the adrenalin appears to be without beneficial effect. Further support to this view is obtained from the history of cases similar to the one here reported in which no status asthmaticus was present, but where the fatal outcome followed the administration of morphine, a powerful respiratory depressant. In the present case, death, which was quite unexpected, occurred 8 hours after  $\frac{1}{4}$  gr. of morphine had been given. Similarly Thieme and Sheldon were of the opinion that in 3 of their 7 cases the administration of morphine was a contributory factor in causing death.

The most interesting feature of this case is the "asthmatic" reaction in the ovarian teratoma. It is all too easy, as Gale and Willis (1944) have recently pointed out, to "identify" organs or parts of organs in a teratoma, and I have consciously refrained from describing the affected tissues as bronchial wall. The correct type of epithelium and abundant mucous glands and muscle would seem adequate grounds for so labelling it, but cartilage is absent, and apart from the muscle it could equally well be nasal mucosa. To term it respiratory epithelium is at least admissible. The significance of the asthmatic reaction is not thereby minimised, as changes similar to those in the bronchial wall have been demonstrated in the nasal mucosa of subjects dying in an asthmatic attack (Kountz and Alexander; Michael and Rowe). The grounds for calling the reaction asthmatic would seem to be adequate. The eosinophilic infiltration, Charcot-Leyden crystals and thickened basement membranes are similar to those in the bronchi, and there are no indications of other

## ASTHMATIC REACTION IN AN OVARIAN TERATOMA



FIG 6 —Bronchus 0.5 cm. in external diameter, showing thickened basement membrane, hypertrophied muscle, one or two bronchial gland acini and intense eosinophilic infiltration. H and E. Dufay colour process.  $\times 165$

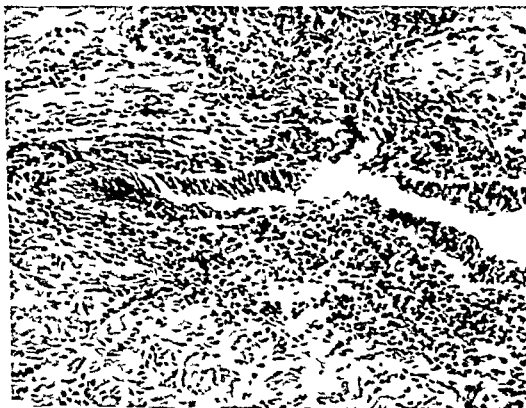


FIG 7 —Respiratory type mucosa from ovarian teratoma showing thickened basement membrane, eosinophil cell infiltration, some unstriped muscle and a portion of a mucous gland. H and F. Dufay colour process.  $\times 165$



possible causes such as necrosis or inflammatory change. Further, the absence of a similar reaction in the adjacent non-respiratory tissues (skin with sebaceous and sweat glands) is very significant. One can only wonder at the high degree of tissue specificity that the case so admirably demonstrates.

The coincidence of two variables, death in an asthmatic attack and the presence of an ovarian teratoma containing respiratory tissue sufficiently well differentiated to show the asthmatic reaction, determined the final picture in this case, and it is interesting to speculate as to the chances of such an association being again encountered. Neither bronchial asthma nor dermoid cysts of the ovary are rare, but the majority of such tumours are nowadays obtained as surgical specimens, and a patient with such a tumour is not likely to be subjected to operation during an asthmatic attack. The available data on the persistence of asthmatic changes is small. Thieme and Sheldon, in 10 cases of asthma dying from a variety of other causes, did not find in the lungs the lesions characteristic of death in an asthmatic attack. Only two showed eosinophilic infiltration of the bronchi, and in the case with the shortest interval between an asthmatic attack and death (15 days), the only pulmonary finding which could be related to asthma was occasional thickening of the basement membranes of some of the medium sized bronchi. This is seen in some cases of ordinary chronic bronchitis, and it would therefore appear unlikely that asthmatic changes in a similar ovarian teratoma would persist long enough for their recognition, if, as is probable, the tumour were removed some time after an asthmatic attack. Nevertheless a careful examination of such tumours and other well differentiated teratomas with this point in view might bring to light further examples, as well as similar appearances in subjects with other allergic diseases or conditions in which specific tissues or organs are affected.

### Summary

In a woman dying in an attack of bronchial asthma there was found in the teratomatous respiratory tissue of an ovarian teratoma "asthmatic" changes similar to those present in the bronchi.

I wish to express my thanks to Dr A. G. Ogilvie for the use of the clinical notes of the case.

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# THROMBOSIS OF THE AORTA IN THE NEWBORN: THREE CASES, ONE WITH INFARCTION OF THE LIVER

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(PLATE XXIX)

Occlusion of the aorta by embolism or thrombosis is rare at any age and especially so in childhood. Rothstein (1935) in a detailed survey of 123 cases of embolism and thrombosis of the abdominal aorta paid special attention to cases under 15 years. He reported a case in a 13-day-old infant and found records of 11 others. Two of these occurred in the 2nd week, two in the 3rd week and the remaining seven between the 9th month and the 12th year. A brief note by Lakin (1934) recorded a case in an 18-day-old infant, Becker and Girgensohn (1939) described cases in infants aged 5 and 16 months, and Bobeff (1937) a case in a 10-year-old girl following diphtheria. Recent reports of cases in adults include 16 reported by Reich (1943) and 3 by Fry (1939).

It is often impossible from the description given to decide whether the occlusion was due to embolism or thrombosis. An embolus resulting from endocardial thrombosis and impacted at the iliac bifurcation with secondary propagation of thrombus upwards, but rarely above the superior mesenteric artery, has often been responsible. Half of Reich's cases were due to embolism. Apart from atheroma, syphilis and aneurysm, little is known of the structural factors contributing to thrombus formation in the aorta.

The present study is based on three cases examined at autopsy. In the first the thrombus was of some duration and the case presented certain interesting problems. In the other two it was more of the nature of a terminal complication and had not produced lesions in other organs.

## CASE REPORTS

### Case 1

*Clinical history.* The infant was a male weighing 7 lb. 6 oz. at birth. Neonatal icterus was marked, but apart from slight vomiting he was normal until the 8th day, when the temperature rose to 102° F. and he appeared



dehydrated. Sulphathiazol and oxygen were given and an oral thrush infection treated, but he remained seriously ill and very dehydrated for three days, though a blood culture was sterile. From the 12th to the 14th day the temperature remained around 101° F., chemotherapy was continued, there was occasional vomiting and the oral thrush infection was refractory. On the 15th day bright red blood was passed per rectum, and this continued for the next three days until death occurred. A vitamin K preparation was given at birth and on the 15th and subsequent days. Healing of the umbilicus was slow, but appeared satisfactory.

### *Post-mortem findings*

*Anatomical diagnosis.* Peculiar focal arteritis of abdominal aorta, with thrombosis extending into the branches of the celiac axis, left renal and inferior mesenteric arteries: infarction of liver and left kidney: patchy hæmorrhagic infarction and ulceration of stomach and descending and pelvic colon. Excessive aspiration of amniotic contents, with histiocytic reaction in alveoli: diffuse atelectasis and undue prominence of alveolar lining cells. Small subarachnoid birth hæmorrhages with localised thrombosis of related meningeal veins. Balanitis. Left subacute otitis media. Unhealed umbilical scar.

*Gross morbid anatomy.* Externally there was no unusual pigmentation and no sepsis except a slight balanitis. A special study of the lower limbs showed no gangrene nor trophic change. The peritoneal cavity contained 4.5 c.c. of blood-tinged fluid. Fibrin and congested subserosal blood vessels were present over the lower half of the colon and on the pelvic peritoneum around the urinary bladder and rectum, and fibrinous adhesions passed to a few of the neighbouring loops of small intestine. The heart was normal and both valvular and mural endocardium were free from thrombi or vegetations. The lungs were uniformly but rather poorly expanded; they were free from any actual areas of consolidation. The thymus was atrophic. The duodenum, pancreas, small intestine, cæcum and ascending and transverse colon were normal. The walls of the descending and pelvic colon and the related mesentery were thickened and œdematous, the subserosal vessels congested and the serosa purple-red and covered with fibrin. This portion of the bowel was dilated and the mucosal surface purple or dark red, except where it was deficient over rather sharply defined ulcers with a dirty yellow floor up to 2 × 1 cm. in diameter. The anal canal and rectum were normal. The stomach, apart from extensive post-mortem autolysis, showed intense but rather patchy congestion of the mucosa. Though the liver was normal in size and its capsule smooth, it felt soft and flabby and large ill-defined areas were seen through the capsule. On section from one-half to two-thirds, and especially the more central and anterior part, was occupied by soft confluent areas where the fine structural pattern was lost. The margins of these areas were not grossly hyperæmic and, though slightly paler and more yellowish grey than the remainder of the organ, they were often difficult to distinguish. The gall-bladder contained bile and the biliary passages were normal. No structural detail was recognisable in the congested spleen; it was only slightly enlarged. The right kidney and both adrenals were normal. Round the left kidney there was slight pericapsular œdema. On section intense congestion of a narrow zone beneath the capsule contrasted with the yellowish white colour of the remainder of the kidney, which was entirely necrotic and structureless. The urinary bladder, prostate and testes were normal. The mouth, pharynx and œsophagus were free from thrush infection. The aorta showed a mass of firm laminated thrombus

extending into the coeliac axis and its branches, the left renal artery and the commencement of the inferior mesenteric artery. In all the iliac branches and in the obliterating hypogastric arteries there was thrombus, but it was less well formed.

### *Microscopical examination*

*Aorta and branches.* At no point did ante-mortem thrombus entirely fill the lumen, but it was most abundant between the coeliac axis and left renal artery. In the thrombus red blood cells had lost their outline and the nuclei of leucocytes were disintegrating. The subjacent endothelial cells were usually deficient and the intima was slightly oedematous, but cellular reaction was limited to swelling of fibroblasts. In the aorta and left renal artery small aggregates of red cells were sometimes found in the intima and between the elastic fibres of the adjacent media. Lying in the media of the aorta and unassociated with any distinctive changes in the adjacent intima or in the thrombus in the lumen, there were a few minute focal lesions (fig. 1). These showed some oedema, infiltration by polymorphonuclear leucocytes and swelling of fibrous tissue cells, but no disruption of elastic fibres. Bacteria could not be demonstrated and there was no inflammatory reaction in the peri-aortic lymphatics.

There was a little recent ante-mortem thrombus, but no embolic material at the iliac bifurcation. Sections excluded propagation from the thrombus in the obliterating hypogastric arteries or spread of infection from the umbilicus along the peri-arterial tissues. The channels of the coeliac axis and commencement of the hepatic artery were not entirely obliterated. The hepatic artery in the porta hepatis contained no thrombus, and it was impossible to determine the completeness of the occlusion of the vessel and its anastomotic channels proximal to this. Occlusion of the left renal artery was complete, but sections of the inferior mesenteric artery failed to establish complete occlusion.

*Liver.* Part of the liver was normal and showed no cellular reaction in the portal tracts and no fatty change in the parenchyma. Near the necrotic areas liver cells showed scarcely any fat vacuolation, but in a narrow zone at the margin they were often slightly atrophic and the sinusoids were distended with red cells. The infarcted areas had an irregular margin because the liver cells around the portal tracts tended to survive (fig. 2). In large areas the liver cells were eosinophilic and had entirely lost their normal arrangement and outline. They were sometimes further disorganised by very small aggregates of red cells, and the nuclei of the sinusoidal lining cells were often preserved when adjacent liver cells had disintegrated. The portal tracts in the infarcted areas were distinct and the bile channels normal. The central lobular veins and the vessels of the portal tracts appeared patent, though the endothelial lining cells were often swollen. Infiltration by inflammatory cells was almost confined to the oedematous tissue of the portal tracts, and aggregates of poly-

morphonuclear leucocytes spread out along the larger tracts often extended beyond the margins of the infarct. Throughout the infarcted tissue a few liver cells at the margin of the portal tracts escaped (fig. 3). Sometimes these appeared to be proliferating, but there was no proliferation of bile duct epithelium.

*Left kidney.* There was almost complete necrosis of cortex, medulla and renal pelvis. In a narrow subcapsular zone there were breaking down polymorphonuclear leucocytes and small confluent hæmorrhages and dilated blood spaces (fig. 4). Internal to this it was still possible to recognise nuclei in a few glomeruli and sometimes in the tubules. Thrombi were present in small radicles of the renal vein and in some of the venous twigs from the upper end of the left ureter and pelvis of the right kidney.

*Stomach and colon.* The changes were essentially similar in both viscera, but they were more marked in the colon. Areas of intense congestion bordered areas where the mucosa was deficient, and often large infiltrations of polymorphonuclear leucocytes invaded the muscle and extended to the serosa. Blood vessels were everywhere congested and ante-mortem thrombi were present in the small veins.

*Veins.* The portal vein and inferior vena cava were free from ante-mortem thrombi.

## Case 2

*Clinical history.* A male child lost 18 oz. in the first 2 days of life. He then had severe diarrhoea, a temperature of 100° F. and inflamed ear drums. There was no response to sulphadiazine and death occurred three days later.

### *Post-mortem findings*

*Anatomical summary.* Congenital aneurysm of ductus arteriosus communicating with aorta: ante-mortem thrombus in thoracic aorta. Non-specific pharyngitis and laryngitis with ulceration: terminal pneumonia: acute bilateral otitis media: acute non-specific œsophagitis.

The pulmonary end of the ductus arteriosus was closed, the vessel wall was normal and no clot projected into the pulmonary artery. About 3 mm. from the pulmonary artery the ductus dilated into an almost circular sac 5 mm. in diameter and this narrowed to 3 mm. to open into the aorta. In this portion the normal intimal mounds were absent.\* Continuous with the dark red blood clot which filled the sac and extending to within 5 mm. of the diaphragm there was a greyish mass of thrombus in the aorta. Microscopically, thick platelet laminae with adherent blood cells and entangled fibrin strands filled the greater part of the lumen, but apart from some proliferation of intimal cells the vessel wall was normal.

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\* For a discussion of the mechanism of anatomical closure of the ductus arteriosus and its anomalies see Jager and Wollenman (1942).

## NEONATAL THROMBOSIS OF AORTA



FIG. 1.—Case 1. Infiltration of media of aorta by polymorphonuclear leucocytes. H. and E.  $\times 200$ .



FIG. 2.—Case 1. At the margin of the infarct, liver tissue survives around the portal tracts. H. and E.  $\times 35$ .

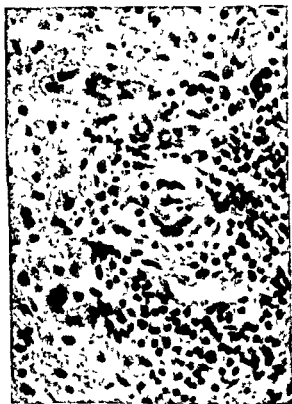


FIG. 3.—Case 1. In the infarcted tissue a few liver cells survive around the portal tracts and there is a heavy inflammatory cell infiltration. H. and E.  $\times 350$ .



FIG. 4.—Case 1. There is intense congestion and inflammatory cell infiltration of the subcapsular zone of the infarcted kidney, with survival of a few renal constituents. H. and E.  $\times 75$ .



## Case 3

*Clinical history.* A male infant died on the 9th day. He was jaundiced, drank poorly and had intermittent diarrhoea from the 2nd day.

*Post-mortem findings*

*Anatomical summary.* Thrush and associated bacterial infection of mouth, pharynx, larynx and œsophagus: bilateral aspiration bronchopneumonia (*Staph. aureus* and *B. coli*). Bilateral renal venous thrombosis with organisation in arcuate and interlobar veins: red and white venous infarcts in both kidneys: organising embolic thrombus in pulmonary arteries. Terminal thrombosis of renal arteries and abdominal aorta.

Thrombus in the lumen of the aorta extended from the cœliac axis to the inferior mesenteric artery. A block of tissue from this region was cut transversely and selected sections from the serial ribbon were stained and examined. Compact thrombus almost occluded the openings of the renal arteries and branching laminae of platelet thrombus extending into the aorta had entangled many red blood cells in a fibrin meshwork. A few endothelial cells had proliferated near the orifice of one renal artery, but there was no reaction in or around the walls of the vessels. Branches of the renal arteries were not occluded and propagation of the thrombus from the infarcted kidneys could be excluded.

## DISCUSSION

Occlusion of blood vessels by thrombosis is usually gradual and often incomplete. When it involves arteries where there is an extensive anastomotic circulation the whole of the area normally supplied cannot become completely ischæmic. In case 1 of the present series the circulation to the lower limbs was not interrupted, but in spite of its systemic and portal blood supply a large part of the liver was infarcted. The results of relative ischæmia of the stomach and colon and of complete occlusion of the renal artery were also illustrated. In cases 2 and 3 the lumen of the aorta was not completely occluded and no structural complications were evident.

Infarction of the liver has been reviewed by Lund *et al.* (1935) and by Pass (1935). They were mainly concerned with the effect of occlusion of the hepatic artery distal to its anastomotic connections. The vascular lesions necessary for infarction have also been investigated experimentally, notably by Cameron and Mayes (1930) in the rabbit. They have shown the importance and complexity of the anastomoses in the arterial blood supply of this organ. There is adequate evidence in man and in the rabbit that occlusion of the systemic arterial blood supply of the liver leads to widespread necrosis. In the present case the extent of the infarcted area and the failure to demonstrate

thrombi in the branches of the hepatic artery in the liver appeared to exclude the carriage of emboli to that organ. The uncertainty as to the patency of the numerous anastomotic arteries of the coeliac axis prevented any estimate of the completeness of the hepatic arterial occlusion. Again the partial occlusion of branches of the coeliac axis and of the inferior mesenteric artery may have secondarily reduced the volume of the portal venous circulation.

A notable feature is the relatively small amount of liver tissue in a condition intermediate between normal and necrotic. In occlusion of the portal circulation the systemic blood from the hepatic artery should prevent necrosis. Atrophy of the liver cells with congestion of the sinusoids results but no true infarction. This is the so-called "atrophic red infarct of Zahn". Zimmerman (1930) discussed the literature very fully and reported a case of widespread infarction of the liver with occlusion of the portal vein in a 31-hour-old baby. He thought it necessary to attribute this outcome to an associated hypoplasia of the hepatic artery. Morison (1944), in discussing umbilical sepsis, attributed to embolic portal occlusion a small solitary area of necrosis surrounded by a relatively wide zone of atrophic liver cells and dilated sinusoids which he encountered in one case. The liver lesions described in a stillborn infant by Palmer (1923-24) were in some respects similar. The essential factor in determining the result of occlusion of any of the vascular channels of the liver is the quality of the blood reaching the liver cells. Toxæmia and circulatory or lung lesions with deficient oxygenation of the blood may influence the outcome as well as the extent and site of the occlusion. Cameron and Mayes failed to produce infarcts in 14-day-old rabbits by hepatic arterial occlusion, but the human infant does not appear to enjoy any such advantage.

Among the six reported cases of occlusion of the aorta in the neonatal period a reasonable explanation was provided only in Rothstein's patient. Fragments of thrombus were found adherent to the endocardium of the left ventricle, and the occlusion was apparently embolic. No such explanation is possible in the present cases. Apart from the absence of any such nucleus in the clot, there was no possible source for a thrombus in the left side of the heart or in the pulmonary veins. A paradoxical embolus can only arise when the foramen ovale is widely open and unguarded by the usual folds.

In cases 2 and 3 local stasis and the occurrence of eddies in the blood stream are the only obvious explanation for the occurrence of thrombosis in this unusual site. In case 2 the wide opening from the aorta into the aneurysmal sac of the ductus arteriosus and the closure of the pulmonary end of the ductus provided a volume of stagnating blood in which thrombus formation might be initiated. In case 3 the extensive venous infarction of the kidneys would produce changes in the blood flow at the orifices of the renal arteries. In case 1 there was no anatomical peculiarity and it appeared improbable

that a primary venous thrombosis had produced uniform pale infarction of one kidney.

The aggregates of polymorphonuclear leucocytes in the media of the aorta in case 1 must represent some form of arteritis and the thrombus formation may have been secondary to this. Many sections were examined. The largest aggregates (fig. 1) showed transitions to much smaller and more diffuse collections, there was no evident infection of the adjacent blood clot and no infection in the peri-aortic lymphatics or loose adventitial tissues around the aorta. The lesions differed from the adventitial aortitis with aortic thrombosis described by Becker and Girgensohn in a 16-months-old baby and attributed by them to spread of infection from the gut to the para-aortic lymphatics. A primary acute aortitis not attributable to an associated or preceding infection of the heart valves or adjacent para-aortic structures is extremely rare. Stumpf (1913) found only nine cases in addition to his own, and Rappaport (1926) reported another. In the present instance, apart from the apparently localised infection of the umbilicus and the healed thrush infection of the mouth, there was no evidence of an infective focus in the body. Chemotherapy may have caused an early resolution of some other infection, such as pneumonia. It may even have masked the full development of some form of infective aortitis.

It is extremely doubtful if the infiltrations in the aorta could be secondary to occlusion of vasa vasorum by thrombus in the lumen of the aorta. The possibility of an arteritis similar to that described by Rich (1942) as resulting from hypersensitivity to sulphonamides, alone or in combination with foreign serum, may be considered. A detailed study in all viscera of arteries of the size usually affected revealed no lesions.

#### SUMMARY

Thrombosis of the aorta was found in three infants in the neonatal period. In two cases stasis, secondary to a congenital abnormality of the ductus arteriosus and to bilateral venous infarction of the kidneys respectively, may have been of aetiological significance. In the other case, which was complicated by infarction of the liver, left kidney, stomach and lower part of the large bowel, a peculiar arteritis was found in the media of the aorta.

I wish to thank Dr F. M. B. Allen for the use of the clinical notes of these cases, Prof. J. H. Biggart for his advice and Mr D. McA. Mehaffey for photography.



The histology of normal human atrio-ventricular valves is detailed in a recent paper (Harper, 1940-41). Fibroblasts constitute the main cellular element of the subendothelial zone. They are most abundant in the distal two-thirds of the cusps and are commonly found adjacent to the surface of collagenous bundles. In adult hearts fibroblasts are for the most part highly differentiated but are less so in the first and second decades of life, in which also smaller cells having the same general appearance are numerous in the connective tissue, especially of the proximal portion of the subendothelial zone. These are regarded as undifferentiated fibroblasts. Scattered throughout this zone, both above and below a superficial elastic layer, are cells which have all the characters of histiocytes. Proximally the subendothelial zone is directly related to the atrial musculature.

### TECHNIQUE

#### *Injection and clearing*

The human hearts were placed in normal saline at room temperature until rigor mortis had passed off. Cannulae were then inserted into the orifices of the coronary arteries and the heart perfused with warm normal saline. The injection masses used were a 3 per cent. aqueous solution of berlin blue or a 2 per cent. aqueous solution of india ink. Rabbit hearts were injected directly through the aorta. A 10 per cent. formalin-acetic mixture was employed as the fixative in most cases. Whole specimens of valves were cleared by Reagan's modification of the Spalteholz method (1926) and permanently preserved in three parts methyl salicylate to one part benzyl benzoate.

#### *Experimental damage to rabbit valves*

This was produced by the intravenous injection of a suspension of "aleuronate". This substance was in the form of a yellowish brown powder almost insoluble in water and composed of amorphous and pseudo-crystalline particles. The suspension used was made up by adding 5 g. of aleuronate to 25 c.c. of cold sterile normal saline and stirring; of this suspension 5 c.c. were injected into the marginal ear vein of the animals before sedimentation had begun.

#### *Vital staining*

Trypan blue was administered subcutaneously in a 2 per cent. solution in distilled water and the animals killed at various intervals after the last injection. This dye was also superimposed on valves previously damaged by aleuronate. Before use the trypan blue solution was filtered and then sterilised by gentle boiling. Blood cultures were made from each animal before the administration of the inoculum and before killing, all with negative results. The amounts of the dye and aleuronate suspension used are given in the accompanying table.

TABLE

Rabbit	Daily dose of trypan blue (c.c.)	No. of injections	Time between last injection and killing
1	2.5	1	12 hrs.
2	2.5	2	2 days
3	2.5	2	10 "
4	5.0	2	2 "
5	5.0	2	10 "
6	7.0	2	2 "
7	7.0	2	5 "
8	10.0	2	2 "
9	10.0	2	10 "

## BLOOD SUPPLY OF HEART VALVES

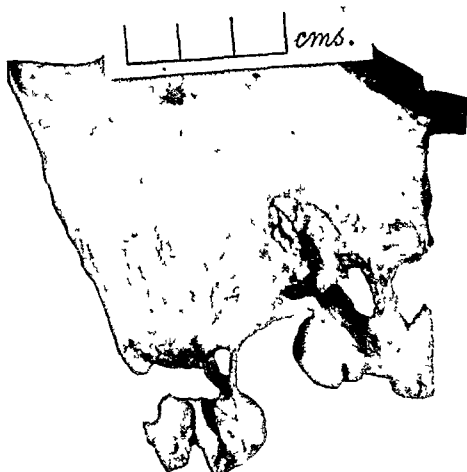


FIG 1—Endocarditis of anterior cusp of mitral Male aged 27 years

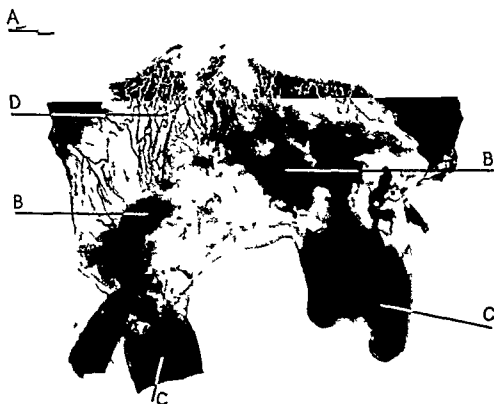


FIG 2—The same specimen, injected and cleared (A) Heavily injected atrial muscle, (B) vegetation, (C) papillary muscles, (D) blood vessels of inflammatory origin



R 10, R 11 and R 12 each received three daily doses of 5 c.c. of aleuronate suspension followed approximately 14 hrs. after the last injection by two daily doses of 5 c.c. each of trypan blue. R 10 was killed 12 hrs. after the last injection of the dye and R 11 and R 12 after 7 and 29 days respectively.

### EXPERIMENTAL OBSERVATIONS

#### *Blood vessels of diseased human valves*

In human valvulitis it is generally admitted that the affected cusps contain a system of blood vessels. All cases of ulcerative type (fig. 1) exhibit a fairly complete vasculature extending from the base of the mitral to the vegetations, many arterioles of large size being observed (fig. 2). Blood vessels extending from the papillary muscles along the chordæ tendineæ occur in most cases of this type. Fig. 4 shows the vascular picture of a case in which the mitral exhibited a small nodular elevation about 3 mm. in diameter. The new blood vessels appear as irregular leashes passing downwards in the direction of the lesion. This vascular pattern gives no idea of time in relation to the disease process and it was at first thought that the new vessels were "growing towards" the lesion. Histological examination of this and similar specimens provided evidence of obliteration of vessels in and around the thickened area. The vasculature, therefore, was "regressing from" the lesion.

The valvular blood vessels in these cases are a new formation; they run for the most part in the subendothelial zone and have arisen in the first instance by proliferation from vessels in the atrial musculature. But it is equally clear that cells associated with their subsequent establishment in the cusps are derivatives of certain subendothelial connective tissue cells which proliferate alongside the advancing capillaries (Harper, 1940-41).

#### *Blood cysts in heart valves*

During this investigation small dark red nodules were frequently found in the atrioventricular valves of the newborn and infants in the first few months of life (fig. 5). They appear to have no pathological significance but are of interest in the present connection since various authors have regarded them as hæmatomata resulting from the obliteration of valvular blood vessels. Others have attributed their formation to blood being pressed from the cavity of the heart into channels or crevices in the young undifferentiated valve. Recent observations favour the latter view (Dow and Harper, 1936-37).

#### *The heart valves of the rabbit*

In work on experimental endocarditis it is necessary to stress the significance of the presence or absence of blood vessels in the normal

cusps of the animals employed. The blood vascular content of the atrioventricular valves of the rabbit and guinea-pig conform to the human type. On the other hand, blood vessels do occur normally in the valves of certain species, *e.g.* cat, dog, sheep, goat, pig and horse (Bayne-Jones, 1917), but these cannot be regarded as analogous to those of inflammatory origin which sometimes occur in man. The rabbit has been used extensively as an experimental animal and many workers have claimed success in the production of endocarditis with injections of certain bacteria. Rosenow (1912) and others believed that the process was one of embolism of blood vessels normally present in the valves, but anatomical studies show that no system of blood vessels pervades the whole extent of the valves in this animal and that the cellular content of the subendothelial zone is also very similar to that of man (Harper, 1938-39).

The intravenous injection of a suspension of aleuronate produces damage to the area of contact on the atrial surface of the tricuspid in the rabbit. Since there are no blood vessels in the cusps themselves this is presumably effected by actual bombardment of the cusps by the aleuronate particles. In these circumstances marked cellular changes take place in the subendothelial zone followed by growth of new blood vessels into this zone (fig. 6). Employing the amounts of aleuronate cited above, signs of commencing proliferation of blood vessels can be observed in 24 hours. The new vessels, at first capillary in size, arise as endothelial outgrowths from pre-existing vascular endothelium of the atrial muscle at the bases of the cusps, and advance as blindly ending sprouts into the subendothelial zone, where by fusion they quickly form a dense network in its proximal part. The form of the growing vascular network is at first that of an indefinite capillary plexus, but as it continues to advance fusion of the vessels in the older parts of the plexus occurs. Simultaneously many of the intervening capillaries retract and disappear and the adult plexus is relatively stable. The new capillary plexus is contained in a relatively thin stratum, the subendothelial zone, and for the most part in one plane; its final form, therefore, is not so dense as might be expected. In the definitive plexus as seen some three months after its formation (fig. 7), it is a constant finding that vessels of capillary order project directly from larger or smaller arterioles before complete ramification of the latter has taken place, and similarly capillaries enter directly into a well developed vein.

### *Vital staining*

Vital staining methods which have been used to demarcate clearly defined groups of cells in other parts of the body can be applied to the study of the subendothelial zone of normal and experimentally damaged rabbit valves. When trypan blue is injected in moderate concentration typical macrophages and undifferentiated connective

## BLOOD SUPPLY OF HEART VALVES

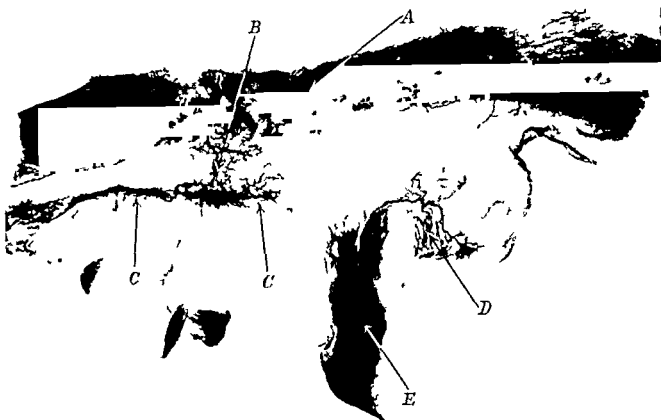


FIG. 3—Endocarditis of tricuspid valve injected and cleared Female aged 52 years (A) Heavily injected atrial muscle, (B) new blood vessels derived from atrial musculature passing to thickened free margin (C), (D) new blood vessels in valve derived from vasculature in a papillary muscle (E)



FIG. 4—Endocarditis of mitral injected and cleared Female aged 36 years Heavily injected atrial muscle and valvular blood vessels of inflammatory origin directed towards nodular thickening

conditions the "sleeve" cells of the chordæ, which occur also in man, doubtless play a part in the establishment of new blood vessels at these sites.

An increased staining property was exhibited by macrophages and to a less extent by undifferentiated cells when the dye was superimposed on damaged cusps (fig. 9). This was presumably accelerated by an increased permeability of the valve tissues, but it is interesting to enquire further into the possible causes of the intense and rapid segregation observed under these conditions. The substance aleuronate is largely protein in nature and it is possible that this substance after injection is accumulated by subendothelial macrophages in particulate or soluble form prior to the injection of the dye. Cytoplasmic inclusions are not readily stained by acid dyes but instances have been recorded (von Möllendorff, 1921). Stained particles of absorbed protein may thus account in part for the increase in number and size of the inclusions observed in the subendothelial cells in these experiments. Nothing in the nature of a generalised upset was observed in the experimental animals following the administration of aleuronate. It was noted, however, that the mitral valve of these animals, while suffering no obvious endothelial damage, exhibited a more intense vital staining, which indicated that in this situation also there was a greater receptivity to the dye.

## DISCUSSION

The blood vascular pattern of the heart valves in the rabbit conforms to the human type. The valvular subendothelial zone in both contains several cell types, many of which under normal conditions are probably non-motile elements playing an important role in the general metabolism of the tissue. In valvulitis many are activated and produce other cell types. This reaction, primarily one of local defence, is secondarily associated with the establishment of new blood vessels within the valve. The new vasculature is clearly part of the inflammatory response. Even in acute cases there is sufficient time for thin-walled capillaries to form. Proof of this is furnished by data recorded by Allen (1939); and in valves from patients suffering from a first attack of rheumatic fever of six weeks' duration or less Gross and Friedberg (1936b) found distant hyper-capillarisation. Harper (1940-41) recorded the early reaction of valve tissue and its relation to new formation of blood vessels in a case of rheumatic valvulitis in a child.

The most probable explanation of the presence of blood vessels beyond their normal limit in human valves is that they have been formed as a result of injury to the cusps. The exact nature of the injury is not fully understood but the work of Gross (1937) clearly indicates that rheumatic fever was responsible for the formation of blood vessels in 44 apparently normal valves.

## BLOOD SUPPLY OF HEART VALVES

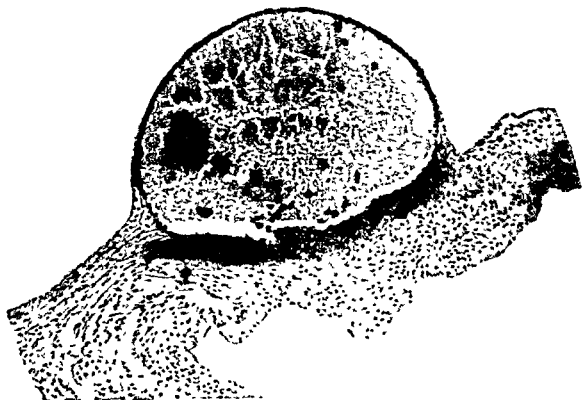


FIG. 5.—Blood cyst on atrial surface of human mitral of a child aged 6 days. Diameter of cyst just under 1 mm.  $\times 55$ .

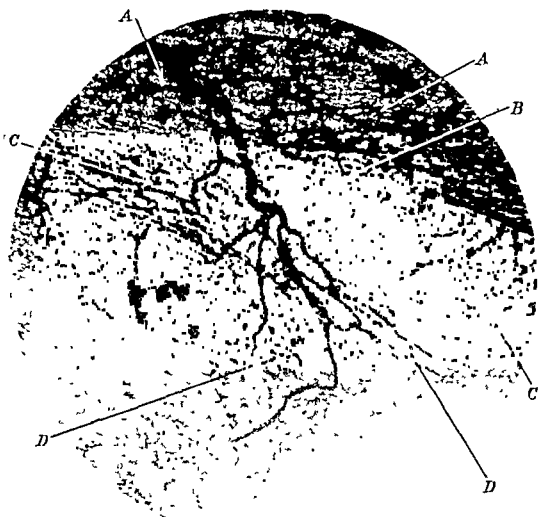


FIG. 6.—Anterior cusp of aleuronate-damaged tricuspid of rabbit, 12 days after injection of aleuronate; injected and cleared. (A) Atrial blood vessels; (B) line of attachment of cusp; (C) and (D) irregular new formations of blood vessels.





Various degrees of healing may occur in valvular endocarditis and thus the extent and persistence of the vascularisation of damaged valves will depend in part on the stage of the lesion.

The question of lymphatics in valves has a direct and important bearing on the problem. Aagaard's monograph (1925) is the most comprehensive treatise on this subject. His findings regarding human valves were entirely negative. It may be, therefore, as Blair (1925) has suggested, that the apparent isolation of heart valves from the blood vascular and lymphatic systems bears some relation to the localised nature of the lesions in septic endocarditis.

Certain results and views with regard to the production of experimental endocarditis, in so far as they concern the present work, may be noted here. Shaw (1904) and Poynton and Paine (1900, 1913) produced it in rabbits by the intravenous injection of large doses of streptococci. Rosenow (1912) produced it in a similar way and considered that the process was one of embolism of blood vessels normally present in rabbit valves. He stated further (1928) that the site of the lesion is determined by a certain quality of the organisms acquired in the individual from whom they were obtained and assumed that streptococcal strains have a selective action on tissues.

Evidence in support of Rosenow was advanced by Borgen (1923) and Haden (1923), but Henrici (1916), Moody (1916), Detweiler and Maitland (1918) and Topley and Weir (1921) have stated that the source of the organism has no influence on its pathogenic effects. Detweiler and Robinson (1916) were of the same opinion and noted further that the deposition of bacteria in the valves seems in some cases to take place on the surface, while in others it appears to be embolic.

During the process of immunisation with pneumococci Wadsworth (1918-19) observed the development of chronic endocarditis in horses and Mair (1923) described a similar condition in rabbits. Dietrich (1926) also observed that endocarditis was more easily produced in animals which had been treated with injections of the same organism, and thought that this was due to sensitisation of the valvular tissues. Wright (1926, 1927) showed that although it is possible to produce endocarditis in this way, it can be done only with great irregularity and he considered that its production may be due to slight injury to the valve rather than to specific sensitisation.

In the production of experimental endocarditis attention has also been directed towards procedures involving the preparatory injection of substances, *e.g.* casein and starch, believed to effect a disposition to the development of endocarditis. Thomson (1935) considered that these substances sensitised the valve tissues. Pfuhl (1929) has described pigmentation, vacuolation and destruction of histiocytes in the valves of animals treated with vaccines and casein, and the work of Semsroth and Koch (1930) indicated that there may be a disturbance

of the detoxifying function of the valve tissue in the genesis of endocarditis.

These results are of interest in the light of the present findings following vital staining of the rabbit valves. Storage of vital dyes can be regarded as a local defence activity, and when loaded with dye particles the cells will presumably become less capable of functioning. Silberberg (1928) has shown that macrophages containing lithium carmine were impeded in their phagocytic activity.

The results of animal experiment and the data available for man indicate that a reticulo-endothelial function can be ascribed to the subendothelial zone of heart valves. In man certain conditions, notably rheumatic fever, activate this zone and in animals the so-called preparatory substances have the same effect. If the exciting cause is continued, impairment of the reticulo-endothelial function takes place and, in species having no blood vessels in the valves, vascularisation of the cusps results. The presence or absence of blood vessels in normal valves has thus an important and direct bearing on the interpretation of experimental results and indicates that care must be exercised in the comparison of the results of experiments on different animals. Thus the statement by Bland *et al.* (1939) that a preparatory substance is unnecessary is probably ill-founded, for the experimental animal which he employed—the dog—is one in which blood vessels can be demonstrated regularly in normal valves. Clearly in such a case primary attack from within the valve would be a likely occurrence and doubtless accounts for the case with which the authors produced endocarditis by the intravenous injection of hæmolytic streptococci. In man and the rabbit on the other hand, primary embolism is impossible. In them the primary attack on the valve is from without, and in the genesis of valvulitis it seems more reasonable to presume that a combination of recent injury and coincident blood infection is necessary, rather than to assume a selective action on the part of the bacteria.

### SUMMARY

1. Normal human heart valves, like those of the rabbit, are largely non-vascular and contain no lymphatic vessels.

2. In diseased valves blood vessels are formed as part of the inflammatory response.

3. Particulate damage to the tricuspid valve of the rabbit, as by the intravenous injection of aleuronate, induces a new formation of blood vessels in its cusps.

4. A reticulo-endothelial function can be ascribed to the sub-endothelial zone of the heart valves.

5. Primary embolism cannot be considered a factor in the genesis of endocarditis in man or the rabbit.

## BLOOD SUPPLY OF HEART VALVES

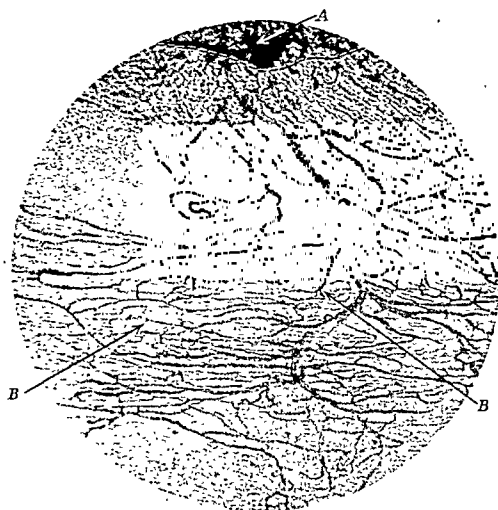


FIG. 7.—Rabbit tricuspid showing definitive new blood vascular plexus 12 weeks after aleuronate damage; injected and cleared. (A) line of attachment of valve; (B) new blood vessels.

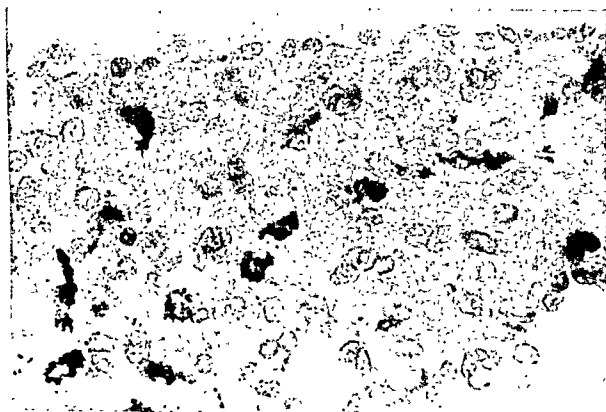


FIG. 8.—Longitudinal section of anterior cusp of mitral. Rabbit 2, 48 hours after injection of trypan blue. Prominent dyo inclusions in macrophages. Carmalum.  $\times 450$ .



## BLOOD SUPPLY OF HEART VALVES

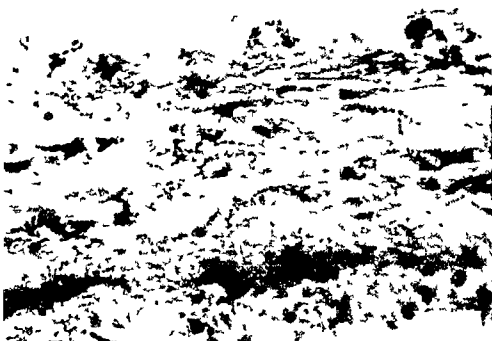


FIG 9 — Longitudinal section of aleuronate damaged tricuspid Rabbit 10 Intense vital staining of cells in atrial subendothelial zone with partial desquamation of endothelium Van Gieson  $\times 400$

FIG 10 — Longitudinal section of anterior cusp of aleuronate damaged tricuspid Rabbit 11 7 days after injection of trypan blue Dye containing cells (chiefly macrophages) near atrial surface Van Gieson  $\times 450$

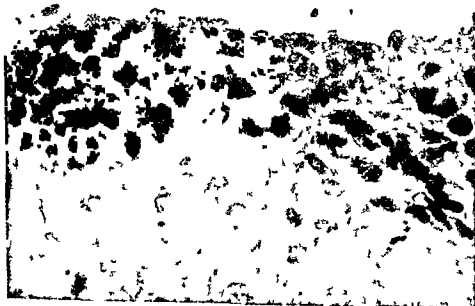


FIG 11 — Longitudinal section of anterior cusp of aleuronate damaged tricuspid Rabbit 12 29 days after injection of trypan blue Compact palisade like thickening on atrial surface and some dye containing macrophages Van Gieson  $\times 450$



6. In many mammals the heart valves are vascularised, so that care must be exercised in the interpretation and comparison of results of experiments aimed at the production of endocarditis in different species.

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616.34—002.1—053.3:576.851.48 (*Bact. coli neapolitanum*)

## ISOLATION OF ANTIGENICALLY HOMOGENEOUS STRAINS OF *BACT. COLI NEAPOLITANUM* FROM SUMMER DIARRHŒA OF INFANTS

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Few investigations appear to have been made on the lactose-fermenting bacteria isolated from cases of the disease of infants variously known as summer diarrhœa, cholera infantum or non-specific gastro-enteritis. The results of such an investigation carried out in parallel with a search for non-lactose-fermenters in these cases are here set out.

The investigation of the lactose-fermenting bacteria followed on the observation of Beavan (1944 and personal communication) that severe cases of diarrhœa often emit a characteristic seminal smell. Winter (1911-12) and Ornstein (1920-21) have commented on the strong semen-like odour given off by certain strains of dysentery bacilli, but dysentery strains are usually absent from typical cases of infantile diarrhœa in this country (Topley and Wilson, 1936). It was considered at first that in our cases the smell might be due to the presence in the fæces of *Proteus vulgaris* (Metchnikoff, 1914; Bertrand, 1914; Costello and Lind, 1939), but on subculturing on nutrient agar the strains of *Bact. coli* isolated from cases, it became apparent that certain of these strains were responsible for the smell. It was noted that the strains in question, identified as *Bact. coli neapolitanum*, showed a delay in the fermentation of maltose. Further investigation showed that organisms with these characters were almost invariably present in cases of severe summer diarrhœa but occurred very infrequently in the fæces of normal infants. It is with these bacteria that the present paper is chiefly concerned.

### *Cases investigated*

The investigation took place between March 1943 and April 1944. There were 51 cases with 20 deaths. In 39 cases the disease was severe and during the summer of 1943 the mortality rate was 44 per cent. The clinical aspects of these cases will be described by Beavan elsewhere, but the usual features were vomiting, diarrhœa and dehydration. The fæces of all cases were examined bacteriologically along with fæces

## RESULTS

*Controls*

Faecal samples from 100 control infants obtained during the March 1943-April 1944 period were examined in the same manner as the specimens from the diarrhoea cases. These infants were hospitalised with the diarrhoea cases and can, therefore, be considered as contacts. Several of them subsequently developed the disease. The figures for normal infants in table I express the actual number of infants from whose faeces the different strains of bacteria were isolated. *Bact. coli neapolitanum* agglutinating to titre with the antiserum and biochemically identical with the strains recovered from the diarrhoea cases was isolated 4 times.

Further control specimens examined for late maltose-fermenters included 17 from normal non-contact infants from a welfare clinic and specimens from 80 adults and older children sent to the laboratory for investigation for various reasons. One strain was isolated from the first group and three from the second; of the last three two came from children aged 2 years suffering from Sonne dysentery; the other strain was from a child aged 5. These three children could be regarded as contacts.

Sixty more faecal specimens from the same number of normal contact infants were then examined for sucrose- and salicin-fermenting strains of *Bact. coli*. Of the 8 strains isolated only one was an agglutinable late maltose-fermenter. In faecal samples from 12 normal breast-fed infants late maltose-fermenters were not found.

*Diarrhoea cases*

The cases are grouped into (a) "Summer" cases, from March 1943 to October 1943 inclusive and those of April 1944 (the weather in March 1943 and April 1944 was exceptionally hot), and (b) "Winter" cases, from November 1943 to March 1944 inclusive.

*Summer cases.* The percentage incidence of the different strains of organisms isolated from the 44 summer cases is shown in table I. Late maltose-fermenting *Bact. coli neapolitanum* was isolated from every case. Forty-two of these strains were agglutinable; two were inagglutinable. As compared with the normal contacts, the frequency of *P. morgani* and the maltose+ strains of *P. vulgaris* was approximately doubled; the frequency of the maltose- strains of *P. vulgaris* approximately trebled; while the frequency of *Bact. coli neapolitanum* rose from 4 to 100 per cent.—a twenty-five-fold increase. The paracolon bacilli and *Bact. sonnei* will be discussed separately.

These findings confirm the observations that have been made on the shift in frequency of *P. morgani* (Lewis, 1911-12), and of *P. vulgaris* (Metchnikoff, 1914) in cases of diarrhoea as compared with normal infants. It was often found that each of the three

lactose-fermenting colonies picked from the MacConkey plate belonged to the same late maltose-fermenting type, suggesting that these organisms had largely replaced the normal *Bact. coli*. It is also

TABLE I

Percentage incidence of organisms in the faeces of normal infants and of the diarrhoea cases

Organisms	100 normal infants	Cases of diarrhoea	
		Summer (44 cases)	Summer and winter (51 cases)
<i>P. morgani</i> . . . . .	15 (12)	32.0 (53)	32
<i>P. vulgaris</i> maltose+ . . . .	6	11.0	10
<i>P. vulgaris</i> maltose- . . . .	30 (33)	54.0 (91.6)	82
<i>Bact. sonnei</i> . . . . .	0	6.8	8
Paracolon bacilli . . . . .	4	4.5	4
<i>Ps. pyocyanea</i> . . . . .	1	4.5	1
<i>Bact. coli neapolitanum</i> . . .	1	100.0	92

The figures in brackets give the findings of Morgan and Ledingham (1908-09) for *P. morgani* during 1908 and of Metchnikoff (1914) for *P. vulgaris* during 1909-13.

noteworthy that in 13 of the diarrhoea cases the faeces were examined before the acquisition of the infection in hospital and are included in the control series. From none of these cases was late maltose-fermenting *Bact. coli neapolitanum* found before the onset of diarrhoea, but this organism was recovered from all after the disease had set in.

*Winter cases.* Seven cases were diagnosed as gastro-enteritis, with two deaths, during the months November to March. The bacteriological findings were irregular. Agglutinable *Bact. coli neapolitanum* was isolated from one case, *Bact. sonnei* from one case; paracolon bacilli biochemically similar to the non-lactose-fermenting variants of *Bact. coli neapolitanum* (described later) but inagglutinable, were isolated twice. The frequency of the various coliforms found in all cases is shown in table I.

### Characteristics of the late maltose-fermenting strains

The biochemical characters of the late maltose-fermenting strains are given in table II. Forty-two of the 44 strains from the cases of summer diarrhoea were agglutinated to titre by the antiserum; 2 strains were inagglutinable.

*Biochemical variants.* Towards the end of the March to October series attention was drawn to certain paracolon-like non-lactose-fermenters which appeared in 8 cases on the desoxycholate and MacConkey plates. Except for the action on lactose the fermentation and biochemical reactions, as shown in table II, were identical with those of the late maltose-fermenting strains. All the strains isolated

plated out. Besides *Proteus vulgaris* and *P. morgani*, the late maltose-fermenting *Bact. coli neapolitanum* (agglutinable) was isolated twice. This evidently shows that not only had the flies access to the infants' dejecta, but also that if the organism is transmissible these insects could act as vectors.

### DISCUSSION

Whether or not *Bact. coli neapolitanum* of type 4988 is ætiologically connected with summer diarrhœa of infants, neither *P. vulgaris* nor *P. morgani* seem likely to fit this role. Neither of these organisms is antigenically homogeneous. The low carrier rate of *Bact. coli neapolitanum* in normal infants supports the idea that its presence in the disease may be significant. Moreover, it is normally abundant in the intestinal tract of the higher animals, but not in man (Winslow *et al.*). If the late maltose-fermenting strains have any ætiological importance it does not appear that they act in the same way as organisms of the dysentery group in view of the absence of gross inflammation of the intestine. The changes in the liver may suggest the absorption of some toxin from the intestinal tract; on the other hand these changes are more probably due to starvation (Dible, 1938-39).

No experiments have yet been carried out to determine whether the organism produces a soluble toxin. Crowley *et al.* in their investigations on neonatal diarrhœa found no evidence of a soluble toxin by injecting fæcal filtrates into mice. Feeding experiments which we made with living broth cultures on small numbers of lactating mice, kittens and rabbits gave negative results. There is some evidence (Light and Hodes, 1943) that neonatal diarrhœa is caused by a virus and it is possible, if summer diarrhœa and neonatal diarrhœa are ætiologically related, that *Bact. coli neapolitanum* may be associated with a virus on the analogy of the association between the virus of hog cholera and *Bact. cholerae suis*.

Nevertheless, it has yet to be shown that these organisms cause the diarrhœa and not that the diarrhœa enables the organisms to flourish. In view of the somewhat hit-or-miss methods of detecting them it may be objected that these strains may always be present in small numbers in normal fæces. This may well be true, and perhaps the best that can be said is that, by present methods, these strains of *Bact. coli neapolitanum* are very hard to find in normal fæces but very easy to isolate in cases of summer diarrhœa.

### SUMMARY

A particular serological type of *Bact. coli neapolitanum* has been recovered from 42 out of 44 cases of summer diarrhœa of infants. The characteristics of these strains are described and their possible ætiological connection with the disease is discussed.

My thanks are due to Sir Alexander Fleming for his interest in this investigation, to Dr F E D Beavan for his valuable co operation, to Dr Doris Stone for reading the MS and to Dr C N Hand and Dr Glynn Jones for specimens. Thanks are also due particularly to Sister M L Chaplin for arranging for the supply of numerous specimens and to Mr J F Stevenson for technical assistance.

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# A COMPARISON OF MODIFICATIONS OF THREE SEROLOGICAL REACTIONS FOR SYPHILIS: WASSERMANN, MEINICKE AND SACHS-GEORGI TESTS

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SEROLOGICAL reactions for the detection of changes due to syphilis should ideally fulfil strict conditions of specificity and sensitivity; that is to say, positive results ought not to be obtained except with sera from syphilitics, and all cases of active syphilis at any stage should be positive. Practically, it is impossible to satisfy these requirements completely. Taking first the condition of sensitivity, it must be remembered that in a recent infection the reaction of the serum does not pass abruptly from the negative to the positive phase; therefore a period of suspicious and weak-positive reactions must intervene. Secondly, when treatment begun at a stage of the disease not too advanced is effective in overcoming the infection, even short of complete sterilisation, the reaction of the serum returns to negative, and this may occur also when latency supervenes spontaneously; this transition is also a gradual one. Accordingly, in cases undergoing vigorous treatment sera may be expected to yield a considerable proportion of less intensely positive reactions, i.e. weak-positive and suspicious. The requirement of specificity is, of course, exceedingly important; it is a prerequisite of any diagnostic reaction that sources of fallacy should be rare or at least precisely defined. In the case of the Wassermann reaction, it has been generally accepted that positive results are practically conclusive evidence of syphilis provided that certain diseases uncommon in this country can be excluded, e.g. yaws and leprosy. Further, it has been concluded on fairly firm grounds that persistently weak-positive results indicate in the great majority of cases a syphilitic infection which is still active or has been active recently. It appears, however, that, apart from syphilis, a tendency to react positively may be manifest temporarily in febrile states, especially in active malaria and acute rheumatic and pneumonic conditions, although as a rule such reactions are weak. For this reason an important precaution is to reinvestigate positively reacting febrile patients after the temperature has become normal. Accordingly, the clinical significance attached to a weak reaction with the serum of a patient under treatment for syphilis differs from that in the case of sera submitted purely for the purpose of diagnosis, since various conditions which are not due to syphilis may cause a tendency towards a positive reaction; and it would be highly inadvisable to stigmatise as syphilitic many patients who are not so infected, even if a small number of syphilitics may be missed in this way. Thus a distinction is drawn between a stringent *diagnostic* positive reaction, which is required in an unknown case before one may say that there is serological evidence of syphilis, and a *therapeutic* positive, which even if weak is acceptable as significant in a known case of the disease and indicates the need for further treatment. This may be expressed



in another way by saying that for diagnosis the results are to be interpreted clinically with a bias toward the negative, while for therapy the bias is toward the positive.

A third desideratum of serological reactions is that progress in the effects of anti-syphilitic treatment of individual cases should be demonstrable by quantitative changes in the reaction tending toward negative. With the Wassermann reaction this is ascertained in one of two ways, either by showing that with consecutive specimens of a patient's serum taken at intervals during treatment the amount of complement fixed has diminished progressively, or, where a single amount of complement is used in the test, by finding that progressively larger amounts of the patient's serum are required to fix this amount. We consider that the latter method is the less desirable, because inactivated human serum may exert either an adjuvant or an inhibitory effect on complement action, and the assessment of these influences is not easy when results with varying amounts of the patient's serum are compared. Of course, both methods are affected by variations in deviability of different specimens of complement (Browning and Mackenzie, 1924). In flocculation tests such as the Sachs-Georgi reaction, varying amounts of serum are added to a fixed quantity of the antigen. A strongly positive reaction is stated to be one in which pronounced flocculation is produced by the serum even in the lower concentrations, while a weak result is recorded when only the highest concentrations of serum lead to marked flocculation, or when no more than incomplete flocculation results with these and lower concentrations. Thus positive sera differ in respect of the smallest amount of serum which produces flocculation and also in the completeness of the flocculation obtained within the range of amounts which flocculate the antigen. Sometimes, also, a zone occurs within the usual range of amounts of serum used, both the highest and the lowest failing to cause flocculation or producing it only weakly, whereas the intermediate quantities flocculate strongly. Further, sera have been met with which did not flocculate at all in the usual range, but showed marked flocculation with smaller quantities; such sera fixed very large amounts of complement in the Wassermann reaction. Accordingly, any abridgement of the range of dilutions of serum (1:2 to 1:64) is undesirable. It is sometimes not clear what quantitative significance should be attached to readings in the flocculation test; but, in general, where the largest amounts of serum produced only weak flocculation and there was no zone, the reaction was considered to be weak or doubtful. In the case of the Meinicke clearing reaction as modified by Robertson and Colquhoun (1939), complete or partial clearing with the largest amount of serum, together with diminution or absence of clearing with the smaller amounts, was read as a weak or doubtful result; on the other hand, complete clearing with the smallest amount of serum, but none or little with the larger amounts, was taken as a strong reaction.

In comparing different serological reactions for syphilis two other features must also be taken into account, which although of much practical importance are of a purely technical nature, namely simplicity in procedure and ease in reading the results. The flocculation and clearing reactions, since they involve only two reagents—the patient's serum and a saline dilution of the antigen—are simpler than the Wassermann reaction, which demands also complement and sensitised red cell suspension, reagents capable of varying behaviour which cannot be completely controlled. On the other hand, satisfactory Wassermann antigens can be prepared with great ease and regularity, whereas the preparation of a satisfactory antigen for the flocculation reaction is difficult, and the antigen for the clearing reaction requires careful standardising. Accordingly, for the latter it may be advisable to rely on a centrally controlled supply.\* In addition,

\* The M.R. antigen of Ford Robertson and Colquhoun may be obtained from the West of Scotland Neuro-Psychiatric Research Institute, 10 Shelley Road, Glasgow, W. 2.

the saline dilution of the Sachs Georgi antigen may vary in its stability when prepared from the same reagents on different occasions, this factor has been found to be quite uncontrollable.

Some forms of the flocculation reaction which can be quickly carried out have been recommended as "exclusion" tests, on the assumption that a serum which reacts negatively in these will not yield a positive result with any other serological test, although positively reacting sera may include some from non syphilitic conditions. Since in diagnostic work generally the proportion of positive reactions is relatively small, time would be saved by applying a reliable exclusion test in the first place, then only those sera which yielded a positive or suggestive reaction would be submitted to a confirmatory test. It will appear from our findings, however, that no single reaction is likely to detect all syphilitic sera, therefore exclusion tests must be accepted with caution.

In the present work a comparison has been made from the above points of view between (a) the Wassermann reaction (W.R.) as carried out by one of us (W. B. K.) with heart cholesterol antigen according to the method described by Browning (Browning and Mackenzie, 1924, Snodgrass and Peters, 1937) and used at the Western Infirmary for the past 25 years,\* (b) the modification of Mehncke's "clearing" reaction (M.R.) introduced by Ford Robertson and Colquhoun (carried out by D. B. C.),† and (c) Mackie and McCartney's (1942) modification of the flocculation reaction of Sachs and Georgi (S.G.R.) (carried out by I. R.)

In recording the Wassermann reaction it has been customary in the past to return four grades of readings—"positive", "weak positive", "suspicious" and "negative". For the purpose of this investigation weak positive and suspicious reactions have been combined in one group as "doubtful", since this term defines precisely their serological significance in the absence of clinical data. The same convention has been adopted for the other tests. "Moderate" and "strong" positive flocculation and clearing reactions have been taken together as "positive". This division of results into three categories—"positive", "doubtful" and "negative"—is felt to be equally fair to all the methods of test. A discrepancy of one grade, i.e. a serum being recorded as positive in one reaction and doubtful in another when tested in parallel, or doubtful in one and negative in the other, is considered as a "minor", while a reading of negative in one and positive in another is a "major" discrepancy. Major discrepancies—errors being excluded—suggest some radical difference in the basis of the reactions. Since the clinical features, controlled in early cases of syphilis by the finding of *sprochates*, must supply the essential foundation for judgment of the reactions, the material has been considered in three separate groups: (i) voluntary blood donors, chiefly women and presumably in average health, who have offered themselves and have been selected after the usual general medical examination, in these the incidence of syphilis might be expected to be somewhat lower than in the general community, (ii) patients attending the V.D. clinic, these being subdivided into (a) untreated cases, not necessarily all syphilitic, and (b) cases of syphilis undergoing treatment, and (iii) patients from general hospital wards, representing a fair sample of the population affected with moderate and severe illness, but largely exclusive of cases of acute specific infections apart from tuberculosis of the lungs and pneumonia.

\* The only change has been that within recent years, including the period of this investigation, sheep red cells have been substituted for ox red cells in the hæmolytic system.

† An acetone and alcohol mixture in the proportion of 3 to 7 is now used instead of absolute alcohol.

results on 8 occasions, the S.G.R. being positive in 9 of the former and none of the latter. The greatest number of discrepancies (153) consisted of the following minor differences: positive M.R. with doubtful W.R. and S.G.R. in 82, and negative W.R. with doubtful M.R. and S.G.R. in 71; a doubtful W.R. with positive M.R. and S.G.R. in 44, and a positive W.R. and M.R. with a doubtful S.G.R. in 29. The large number (179) giving a doubtful W.R. among the discrepancies in this series (16 per cent.) were interpreted as "therapeutic positives", in accordance with the principle stated above.

It is clear that the M.R. and to a less extent the S.G.R. are more persistent than the W.R. under treatment, although this is not invariably the case. Thus there is no constant parallelism between the strength of the three reactions when sera from syphilitic patients undergoing treatment are tested on repeated occasions. Observations made by one of us (W. B. K.) on the behaviour of protein fractions of the serum in the W.R. and S.G.R. suggest that at least a partial explanation of the discrepancies is that constituents of individual sera may interfere with or augment each of the reactions to an unequal degree.

#### *General hospital routine group*

In a large proportion of cases entering general medical wards the blood is submitted for the serological test as a routine; these constitute the majority of the present series, but a much smaller number coming from surgical wards were selected because of suspected syphilis. In contrast with the treated V.D. series, anti-syphilitic treatment is not likely to have been administered recently to any appreciable number of these patients.

**Series I** (902 cases). There was agreement in over 93 per cent. Of the 56 discrepancies (6.2 per cent.), 9 (1 per cent.) were major; of these, 2 gave a positive W.R. unsupported by either of the other two reactions, while a positive W.R., together with either a positive M.R. or S.G.R., the other being negative, occurred in 1 case each. The largest group of minor discrepancies (12) consisted of a doubtful W.R. with negative M.R. and S.G.R. and next a doubtful M.R. with W.R. and S.G.R. negative in 11.

**Series II** differed from the rest, since only the W.R. and M.R. were compared (table II.). In 4348 reactions (including the cases of series I) 95.4 per cent. agreed; this corresponds closely with the agreement of all three reactions in series I. Of the 200 discrepancies only 19 (0.43 per cent.) were major, the W.R. being positive in 11 and the M.R. in 8. Of the 181 minor discrepancies (4.2 per cent.), over a third (72) consisted of a doubtful W.R. with a negative M.R., about a quarter (47) were a negative W.R. with doubtful M.R., a fifth (35) were a doubtful W.R. with positive M.R., while under one-sixth (27) were a positive W.R. with doubtful M.R.

In these routine hospital series, again, one form of reaction may

be positive and the other negative in a small proportion; but, contrary to the findings in the treated V.D. series, there is no marked preponderance of positive results with the M.R. or S.G.R. over the W.R. Inquiry into the history and clinical condition in the cases presenting serological discrepancies in this group unfortunately yielded little information of value. In a small number of febrile patients in whom the W.R. was positive or doubtful, subsequent examinations of the serum after the pyrexia had subsided gave negative results. In such cases the M.R. and S.G.R. were negative throughout.

### SUMMARY

The W.R., M.R. and S.G.R., each in modified form, have been compared in three groups of cases: (i) voluntary blood donors in average health, (ii) patients attending a V.D. clinic and comprising (a) untreated cases and (b) cases of syphilis undergoing treatment, and (iii) hospital patients in general medical and surgical wards. Results have been classified as "positive", "doubtful" or "negative"; discrepancies with the same specimen of serum where one test was positive and another negative are termed "major", otherwise they are regarded as "minor".

In group (i) (blood donors) the results correspond in 99.3 per cent. In group (iii) (general hospital patients) discrepancies amount to about 6 per cent. (1 per cent. major), while in group (ii) (V.D. clinic) there are 14.9 per cent. of discrepancies (2.9 per cent. major) among untreated cases and among treated syphilitics 39 per cent. (4.2 per cent. major). In a small number of pyrexial patients in group (iii) the W.R. was positive or doubtful but became negative when the fever disappeared; in such cases the M.R. and S.G.R. were negative throughout. In patients undergoing treatment for syphilis the M.R. and, to a less extent, the S.G.R. tend to persist in the positive state longer than the W.R. The preponderance of positive results in the M.R. or S.G.R. over the W.R. is much less pronounced in the other series.

It must be emphasised that in all groups major discrepancies occur, *i.e.* one reaction is positive where one or both of the others are negative, although this is much more common in V.D. patients and particularly in treated syphilitics than in any other group. Thus more complete information as to the presence of syphilis is obtained by carrying out two reactions simultaneously, especially the W.R. and one of the other two, particularly the M.R. The latter is recommended for use in parallel with the W.R. because of the ease of its performance and of reading the results. None of the three reactions singly can be relied on as an "exclusion" test. Accurate preliminary standardising of the "antigen" reagent is essential for the M.R.; the preparation of one which is suitable for the S.G.R. presents considerable difficulty; that for the W.R. is simple to prepare.

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## SHORT ARTICLES

547 9 (Penicillin) 576 8 093 6

### A SIMPLE AND CONVENIENT METHOD FOR THE ASSAY OF PENICILLIN

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The introduction of penicillin as a therapeutic agent and the consequent demand for laboratory control of its administration has thrown an increased strain upon the laboratory. The following 'depth test' was therefore elaborated and has been in use in this laboratory for over six months with satisfactory results. The method has been used for estimating the yield of penicillin in cultures of the mould, for gauging its excretion in the urine of penicillin treated cases and for checking the unitage of penicillin used therapeutically. It is not recommended as a method for estimating the bacteriostatic level of sera.

For the tests an agar of low nutritive quality is recommended. The medium employed in this laboratory is the phosphate peptone Lenceo agar elaborated by Stevens and Pope (personal communication) and employed by them in the ring plate method for the assay of penicillin. It is prepared as follows.

#### *Agar medium*

- 10 g peptone (Lvans)
- 3 g Lenceo
- 2 g sodium chloride
- 10 g sodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ )

The above constituents are dissolved in 500 ml of distilled water and placed in a cylinder with 30 g of agar and the volume made up to 2 litres with 1 per cent sodium phosphate at pH 7.0. This is well mixed in a flask and the pH adjusted to 7.0 if necessary. The medium is then autoclaved for half an hour at 15 lb pressure, filtered into bottles in approximately 100 ml volumes, autoclaved for 10 mins at 10 lb pressure and stored at room temperature until required.

With this buffered medium there appears to be no need to adjust the pH of material for test, at any rate over a range from about pH 4.0 to 8.0. It is advised, however, that before bottling the medium for storage, the strength of gel should be ascertained according to the method of Jenkins (1921) and suitable dilution carried out at this stage if required.

For convenience a bottle of agar is melted and narrow test tubes of about 8 mm internal diameter—Unifac heavy  $5 \times 7/16$  in. tubes are suitable—each receive 1.5 ml of the medium. These tubes are stored and when required a number are melted, cooled to  $45^\circ\text{C}$  and each seeded with one drop of a  $1/300,000$  dilution of a 24 hour broth culture of a penicillin sensitive staphylococcus, the culture having been standardised to match no. 1 of Brown's scale.

before dilution. Before being set upright in cold water, the agar is run up the sides of the tubes to remove the condensation water and the contents of the tubes thoroughly mixed to give even distribution of the cocci. Tubes so prepared can be kept at 4° C. for up to four days without detriment.

When using the seeded tubes it is necessary to dilute the fluid under investigation so that the final concentration of penicillin will fall between 0.5 and 2 units per ml.; with culture fluids and with urine, a range of dilutions of 1:50, 1:100 and 1:200 in peptone water is sufficient for most purposes; with penicillin solutions for therapeutic purposes further dilution will of course be necessary. A seeded tube is required for each dilution. On top of the agar in the tube, 0.25 ml. of the dilution is pipetted. The tubes are then placed upright in racks and incubated at 37° C. for 16-24 hours.

When the tubes are taken from the incubator, the distance between the bottom of the agar meniscus and the uppermost colony visible to the naked eye is measured in mm. With the staphylococcus in use in this laboratory the depths of inhibition of growth in repeated tests with Standard penicillin have been:—2 units, 10 mm.; 1.5 units, 9 mm.; 1 unit, 7.5 mm.; 0.5 unit, 6 mm.

It must be emphasised that the tubes should be incubated at once after the addition of the diluted penicillin; if this is not done, the balance between rate of growth of the staphylococcus and rate of diffusion of the penicillin will be altered. This was shown experimentally by leaving tubes, prepared as stated, for 24 hours at 4° C. before incubation at 37° C. The depths obtained were:—2 units, 17 mm.; 1.5 units, 15 mm.; 1 unit, 13 mm.; 0.5 unit, 12 mm.

Narrow tubes are used for the test, and are well spaced in the racks during incubation so that they rapidly attain the temperature of the incubator. If desired, water-bath incubation can be substituted, but the depth to which inhibition occurs must be checked by control tests using Standard penicillin, as the degree of inhibition is slightly less when water-bath incubation is used.

While stress has been laid upon immediate incubation of the tubes, experiments have shown that the volume of diluted penicillin added, the strength of the agar used and the diameter of the tubes—always keeping the size of inoculum per ml. of medium constant—do not alter the readings obtained to any extent. It is necessary, however, to perform a number of tests using Standard penicillin with the chosen staphylococcus so that a reliable base line for unitage estimation can be obtained. The unitage of material under test is, of course, obtained by multiplying the unitage of the diluted material by the dilution factor, *i.e.* 50, 100 or 200 as the case may be. The results are of sufficient accuracy for most routine purposes.

### Summary

A simple "depth test" method for the assay of penicillin using an agar of low nutritive quality is described. Fluids containing penicillin are suitably diluted and an aliquot of each dilution run into the tubes of agar, the depth of inhibition obtained after incubation being the index of unitage.

The author wishes to thank Drs Stevens and Pope of the Wellcome Physiological Research Laboratories for permission to publish the details of the agar medium employed.

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615.778 (Penicillin)—015.25:546.72

## THE INACTIVATION OF PENICILLIN BY METALLIC IRON

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In view of the danger of contamination of penicillin during its manufacture by organisms which are insensitive or only slightly sensitive to its action, it is essential that samples of the dried powder should be tested for sterility. In the first test which was commercially used in this country, the powder was dissolved in sterile distilled water to make a solution containing 1500 units per c.c., 2 c.c. of this solution being placed in 100 c.c. of papain digest broth for aerobic cultivation, and a similar quantity into a minced meat medium for anaerobic culture. The samples of broth were incubated for 28 days and examined at 5-day intervals for bacterial growth. At the end of 28 days they were tested to ensure that all the penicillin had disappeared. Three more rapid methods have since been reported. Lawrence (1943) showed that penicillin could be inactivated by taka diastase, 2 c.c. of a 1 per cent. solution inactivating 2400 units of penicillin in 48 hours. This was used as a basis for a 7-day sterility test employed by the Winthrop Co. Harper (1943) showed that a useful penicillinase could be extracted from some strains of *Bact. coli* and Ungar (1944) prepared a more constantly active penicillinase from a member of the *subtilis* group of bacteria. When this organism was grown in papain digest broth for 3 days and the fluid Seitz-filtered, 1 c.c. of a dilution of 1:250 of the culture fluid inactivated 50 units of penicillin in 4 hours, the quantity of penicillinase produced depending on the strength of the surface pellicle of the original growth.

In an effort to produce a simpler and more constant method of inactivating penicillin, the effect of metallic iron was investigated.

*Experimental*

The medium employed was a 0.2 per cent. sloppy agar containing 1 per cent. dextrose and 1 per cent. "Bacto" peptone; it was tubed in 50 c.c. quantities and will support the growth of both aerobic and anaerobic organisms. Solutions of penicillin were used at a strength of 10,000 units per c.c., 1 c.c. being added to 50 c.c. of medium. The penicillin content of the medium was tested each day against the Oxford H staphylococcus, using the agar cup method of Fleming (1942).

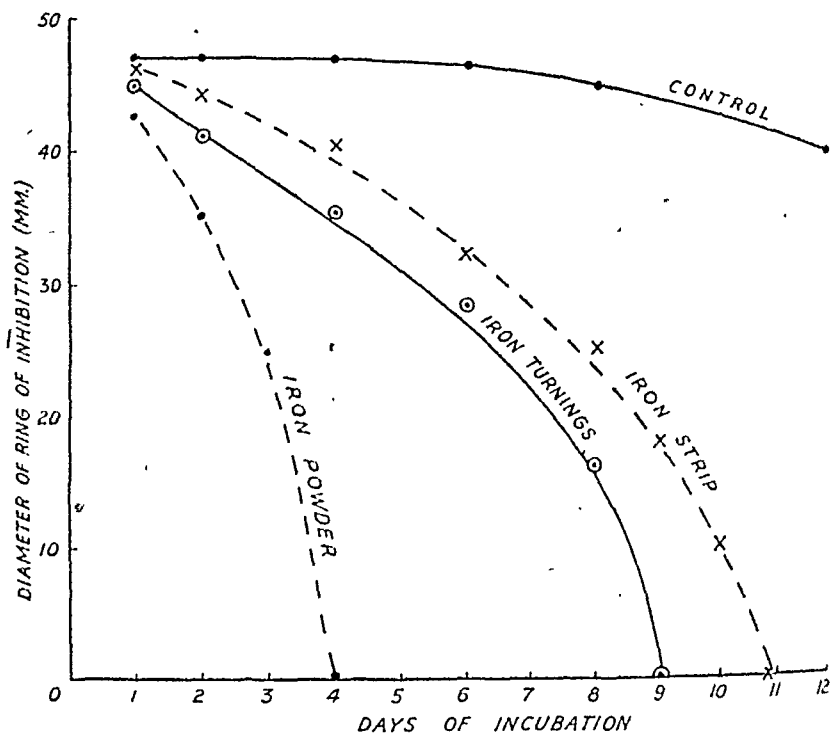
Metallic iron was added (a) as a metal strip, 25×3 mm., of "sheet iron", gauge no. 26, similar to the material used in the anaerobic cultivation of organisms, (b) as 1 g. of "turnings" each 1 mm. in width and about 3 cm. in length, to provide a rather larger surface exposed to the penicillin, and (c) as 1 g. of pure iron powder prepared by the reduction of pure ferrous sulphate in an atmosphere of hydrogen. All the preparations of iron were sterilised by dry heat at 150° C. for 1 hour.

*Results*

The inactivation of penicillin was considerably accelerated by the presence of metallic iron, iron powder being more active than metallic turnings, which in turn are more rapid in action than a metallic strip.



The mean of five experiments is shown in the figure. One g. of iron powder regularly inactivated 10,000 units of penicillin in 3-4 days, the medium at the end of this interval readily supporting the growth of the Oxford H staphylococcus.



The effect of inactivation of penicillin by metallic iron.

### Summary

1. A simple method of inactivating penicillin by means of metallic iron is described.
2. The greater the surface of metallic iron exposed to penicillin, the more rapid is its action.
3. One g. of iron powder will inactivate 10,000 units of penicillin in 4 days.

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576.8 097 576 858 13 (Vaccinia)

THE ANTIGENICITY OF VACCINIA VIRUS  
INACTIVATED WITH ALCOHOL

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No antigen with satisfactory immunising properties has been obtained from inactivated vaccinia virus. Bland (1932) studied the relative immunising power of virus preparations inactivated by heat, phenol and formalin. His paper contains an account of attempts by other workers to obtain a satisfactory antigen. Bland's results in the immunisation of rabbits and monkeys were very disappointing but, when guinea pigs were used, the results were more encouraging. In two of the three guinea pig experiments, however, two successive tests for immunity were employed, the first with a weak dose of virus, the second with a stronger test dose, it may be that the living virus used in the first test, although it produced no obvious lesions contributed to the apparent partial immunity at the later test. There was no definite evidence that the phenol or formalin inactivated virus was more effective than the heat treated antigen. Parker and Rivers (1936) made an extensive study of the immunising activity of purified elementary body suspensions of vaccinia inactivated by 0.3 per cent formaldehyde and of virus free extracts of vaccinia. They found that humoral antibodies and a certain degree of resistance to infection, probably not enduring, could be produced by repeated injections. The production of precipitins and agglutinins did not parallel that of neutralising antibodies. It appeared worth while to investigate the antigenicity of virus inactivated with ethyl alcohol. If a satisfactory basal immunity could be established with this antigen, it might be used in conjunction with later vaccination with active virus to reduce the malaise and risk of encephalitis that follow primary vaccination in older children and adults. It was decided to use rabbits rather than guinea pigs as test animals, since they can be readily immunised with active virus and should show an adequate response to any antigen likely to be effective in man.

*Methods*

*Inactivation of virus* Preliminary observations were made to determine the concentration of ethyl alcohol and of acetone which would completely inactivate an elementary body suspension (e.b.s.) of vaccinia. The treated material was titrated both intracutaneously and by scarification on rabbits in parallel with the untreated e.b.s. In addition, 0.5 ml. of each undiluted preparation was inoculated intratesticularly into another rabbit, this animal was killed on the 5th day, the testes were removed, examined for macroscopic lesions, minced and extracted with an equal volume of saline. The resulting extract was inoculated into a normal rabbit intracutaneously and by scarification. Completely negative results from the transcutaneous intracutaneous and intratesticular tests were accepted as proof of inactivation of the virus. Our observations indicated that exposure for 3 hours at 14°C. to both 75 and 50 per cent concentrations of either alcohol or acetone inactivated the virus but that 25 per cent of either solvent was ineffective. It was decided to use 50 per cent alcohol in our experiments and, after inactivation, to dilute the material to a final concentration of 25 per cent alcohol before injection.

*Preparation of elementary body suspensions* In two preliminary experiments elementary body suspensions prepared by the methods described by Henderson

and McClean (1939) and dried from the frozen state were used. In the third experiment, in which it was decided to use a very large dose of inactivated virus, successive preparations were made from vaccine pulp by differential centrifugation (Macfarlane and Salaman, 1938). These were inactivated with 50 per cent. alcohol and maintained at 37° C. overnight in order to destroy as many contaminating micro-organisms as possible; they were stored at -10° C. in 50 per cent. alcohol until sufficient material had been accumulated to permit a final tenfold concentration by centrifugation before injection. Centrifugation was continued for six hours in an angle centrifuge, after which the supernatant fluid was water-clear. Each preparation was titrated for vaccinia potency before exposure to alcohol. All immunising injections were given subcutaneously in a final concentration of 25 per cent. alcohol.

*Tests of immunity.* During immunisation the rabbits were kept in a house a quarter of a mile from the vaccine lymph laboratories and animal houses and they were cared for by an attendant who had no contact with animals infected with vaccinia. Tests for immunity were carried out by titration of untreated e.b.s. intracutaneously and of a standard vaccine lymph by scarification on each rabbit three weeks after the last immunising dose, in parallel with comparable titrations on normal rabbits. The apparent titre of active virus in both the immunised and normal rabbits was recorded and the relative rate of evolution of the lesions noted. In addition the sera of the immunised rabbits, obtained before the test for immunity, were examined for virus-neutralising potency in parallel with their pooled serum before immunisation and with a sample of hyper-immune anti-vaccinia rabbit serum.

### Results

Two preliminary experiments will be briefly summarised and the results of a third presented in more detail.

**Experiment 1.** Three rabbits received two doses of inactivated virus each equivalent to 50,000 minimal infecting doses (m.i.d.) at three weeks' interval. In the tests for immunity the endpoint of virus activity was a tenfold dilution higher in the control than in the treated rabbits and the lesions evolved more rapidly in the treated animals. With a twofold dilution of normal rabbit serum a standard lymph produced a confluent eruption at  $10^{-3}$ , but with a similar dilution of sera from the treated animals confluent eruptions were not obtained at a dilution of the virus higher than  $10^{-1}$ .

**Experiment 2.** Three rabbits received a single dose equivalent to 100,000 m.i.d. of virus and another three received two similar doses at a three weeks' interval. Tests after a single dose revealed no significant immunity in two out of three rabbits and the serum from six animals after a single dose showed no significant virus-neutralising power. In two of the three rabbits that had received two immunising doses, test virus showed from a tenfold to a hundredfold decrease in titre as compared with normal rabbits. Moreover serum from these rabbits now caused a hundredfold reduction in apparent titre of virus mixed with it.

It was considered that these results, though inconclusive, justified a third experiment in which much larger immunising doses were used.

**Experiment 3.** A series of preparations was made from vaccine pulp by differential centrifugation; the least potent preparation produced lesions after intracutaneous injection of 0.2 c.c. of  $10^{-6}$  or  $10^{-6}$  dilution and by scarification at dilutions from  $10^{-4}$  to  $10^{-5}$  and some were a hundred times more potent. After treatment with alcohol the pooled preparations were concentrated a further ten times by centrifugation and tested for bacteriological sterility; these tests revealed the presence of Gram-positive bacilli of diphtheroid type and some Gram-positive cocci. Two rabbits each given

5 ml of the concentrated preparations subcutaneously and observed for a fortnight did not develop any obvious lesions. In addition two rabbits received 0.5 ml of each preparation intratesticularly, when they were killed on the 5th day no evidence of orchitis was found and testicular extracts failed to produce lesions on intracutaneous and scarification tests, nor were organisms seen in stained films. This elementary body suspension was used for the immunisation experiment.

Six rabbits each received a subcutaneous injection of 10 ml of the concentrated preparation, followed three weeks later by a second dose of 14 ml. Thus each rabbit received a total inoculum of inactivated material equivalent to  $1.2 \times 10^9$  minimal infecting doses of virus. About three weeks later the rabbits had developed apparently painless and freely movable abscesses varying in diameter from 1 to 5 cm at the site of injection, when these were evacuated it was found that the caseous pus contained diphtheroid organisms similar to those detected in the inoculum. It was considered that these slowly developing abscesses were not likely to have interfered with the immunising activity of the preparations.

Three weeks after the second dose the six rabbits were tested for their immunity, together with three normal rabbits. The results, summarised in table I, indicate that the treated rabbits resisted from ten to a thousand minimal

TABLE I

*Titration by intracutaneous injection of an elementary body suspension and by scarification of vaccine lymph in six treated and three normal rabbits*

Intracutaneous titre of elementary body suspension	Scarification titre of lymph	
	Confluent lesions	Discrete papules
Treated rabbit 1 $10^3$	$10^3$	$10^4$
" " 2 $10^3$	$10^2$	$10^3$
" " 3 $10^3$	$10^2$	$10^{-3}$
" " 4 $10^3$	$10^{-1}$	$10^3$
" " 5 $10^4$	10	$10^3$
" " 6 $10^3$	$10^1$	$10^{-3}$
Normal rabbit 1 $10^4$	$10^4$	$10^{-5}$
" " 2 $10^{-5}$	$10^4$	$10^3$
" " 3 $10^4$	$10^4$	$10^5$

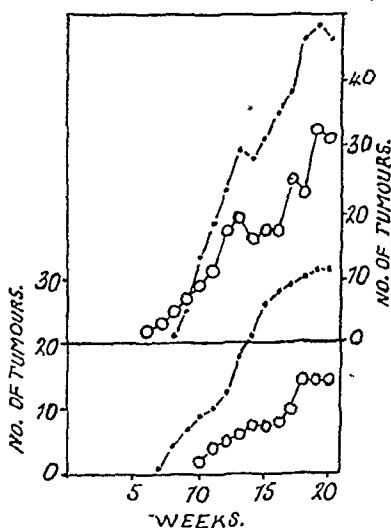
The figures indicate the highest dilution of the test material which produced characteristic vaccinal lesions

infecting doses of virus. Moreover the lesions that developed in the treated animals were generally more hemorrhagic on the second and third day than those in the normal rabbits and were tending to dry up and fade by the fifth day when the lesions in the controls were maximal.

Pools of equal volumes of serum from each rabbit obtained before and 10 days after immunisation were tested for virus neutralisation, together with a sample of glycerinated "hyperimmune" serum. These tests were done in two ways by testing a constant amount of virus against rising dilutions of serum and *vice versa*. Both methods gave similar results and only the former type of test is illustrated in detail in table II. It is evident that the serum from the treated rabbits contained some virus neutralising power but that this

In the first experiment six mice were painted at midday on the right flank with 0.03 c.c. of 0.3 per cent. benzpyrene in acetone over an area of  $2.5 \times 1.5$  cm. and similarly at midnight on the left flank: this was done on three successive days and nights. The flanks were then painted thrice weekly for 20 weeks with 1 per cent. croton oil in acetone. The lower panel in the figure gives the tumour yield and shows that about twice as many tumours resulted from the midnight paintings.

In the second experiment a single painting of benzpyrene was used instead of three. The number of mice was increased to twenty-four, because the yield of tumours from single paintings is much less than from three. In half the mice the right flank was chosen for midnight painting, in the other half the left flank: further, in half the mice the midnight painting was first made, in the other half the midday. Lighting conditions were controlled by placing the



The graphs show tumour yield plotted against weeks after benzpyrene painting: circles = midday, dots = midnight painting. The lower panel refers to the first, the upper to the second experiment. The upper panel sometimes shows a decrease in the number of tumours, due to the well known occasional disappearance of small warts.

mice after midday painting for a few hours in the dark, so as to imitate the lighting conditions after midnight painting. No differences were referable to these varied conditions. The results are shown in the upper panel of the figure: at 20 weeks there were 47 tumours on the "midnight" flanks and 31 on the "midday".

In order to provide a more complete record, the number of tumours at 20 weeks in each mouse is given as follows, the figures for the "midnight" flanks first. In the first experiment 16,9 : 8,1 : 4,3 : 2,1 : 1,0 : 0,0 : in all 31 and 14: in the second experiment 6,3 : 5,2 : 5,2 : 5,2 : 3,0 : 3,2 : 3,2 : 2,0 : 2,1 : 2,1 : 1,0 : 1,0 : 1,0 :—1,1 : 1,1 : 1,1 : 1,1 :—0,4 : 3,6 : 1,2 :—0,0 : 0,0 : 0,0 : 0,0 : in all, 47 and 31.

The chance that there is no real difference between day and night painting and that the observed figures are due to random sampling is about 1 : 500; or if consideration be given only to whether there are more or less tumours on the "midnight" flanks (17 positive, 3 negative, 4 alike and 5 with no tumours) then the chance is about 1 : 400.

In a third experiment painting with croton oil five times in ten days preceded the single painting with benzpyrene, since it was found that this did not disturb the diurnal mitotic variation and it favours a large tumour yield. Nevertheless the number of tumours was small using 12 mice, there were only eight tumours on the "midnight" flanks and four on the "midday".

These experiments are published in the hope that they will be repeated elsewhere. The results are so revolutionary that one is suspicious there is some factor overlooked and not controlled. I am aware of one only, the temperature at which the mice were kept was colder at night than by day. If the results are confirmed then the specific cellular change must be restricted to so narrow a limit of time and cell state that its elucidation is greatly facilitated.

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618.19—006 37—018 725

ON THE 'PINK' EPITHELIUM OF THE CYSTIC BREAST  
AND THE STAINING OF ITS GRANULES

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(PLATES XXXV AND XXXVI)

The text books of pathology and many of the publications on the breast discuss the so called pale or pink epithelium without so much as mentioning the granules present in many of its cells. Some writers refer to the occasional "granularity" of these cells but it is clear that this ambiguous term is being used to describe the state of dispersion of the cytoplasm as seen in the stained section and does not always signify the presence of those clearly defined intracytoplasmic structures which we commonly call granules. Actual granules are described by Schultz (1933) and he illustrates these by coloured drawings as poorly defined, moderately eosinophilic bodies or as well defined dark blue granules as shown by the ferrocyanide reaction. It seems probable that the apparent lack of interest in these granules may be traced to the difficulty of showing them up distinctly by any of the usual stains, and on this point my own experience may be worth telling. Some years ago sections from a case of cystic disease of the breast showed with haemalum and eosin the appearance of granularity in the pale cells, further sections were then stained by haemalum and phloxin (phloxin 0.1 g, phenol 2 g, 50 per cent ethanol 50 c.c., glacial acetic 0.25 c.c., 10 per cent calcium chloride \* 1 c.c.), followed by differentiation with tartrazine in cellosolve. The granularity now revealed itself as sharply stained granules present in great number (figs 2 and 3). Since then, the same method (Lendrum 1939) has been applied to other cases without ever again bringing about the same brilliant result. Recently, however, a new staining

\* The addition as originally suggested by Conn and Holmes (1928), of calcium chloride to aqueous solutions of fluorescein dyes improves their staining power and can rejuvenate a solution that has lost its power.

method has been tried which is easy to perform, seems to show more granules than the test for iron, and has revealed, in all of twenty cases of cystic disease, that wherever there is pale epithelium some of it contains granules.

### *Method*

The method is a slight modification of that recently published by McLetchie (1942-44), who showed that in staining the pituitary it was possible to obtain a strong colouration of the basophil granules by acid fuchsin if the section were first mordanted with iodine and then treated with alcoholic phosphotungstic acid before applying the fuchsin. This, so far as I know quite novel, procedure opens up interesting possibilities in the staining of granules in other sites. All the tissues used here were fixed in formol-sublimate solution (Lendrum, 1943) and put through to paraffin by the routine ethanol-chloroform method (Lendrum, 1941).

Stain with hæmalum for the usual time.

Stain for 1-2 mins. in aqueous 1 per cent. fast green—FCF (Vector)—containing 0.5 per cent. glacial acetic.

Rinse in water and immerse in Lugol's iodine for 2 mins.

Flush with 95 per cent. ethanol.

Immerse in 2 per cent. phosphotungstic acid in 95 per cent. ethanol for 2 mins.

Rinse briefly in water.

Stain in carbacid fuchsin (CAF) for 2-6 mins., controlling microscopically.

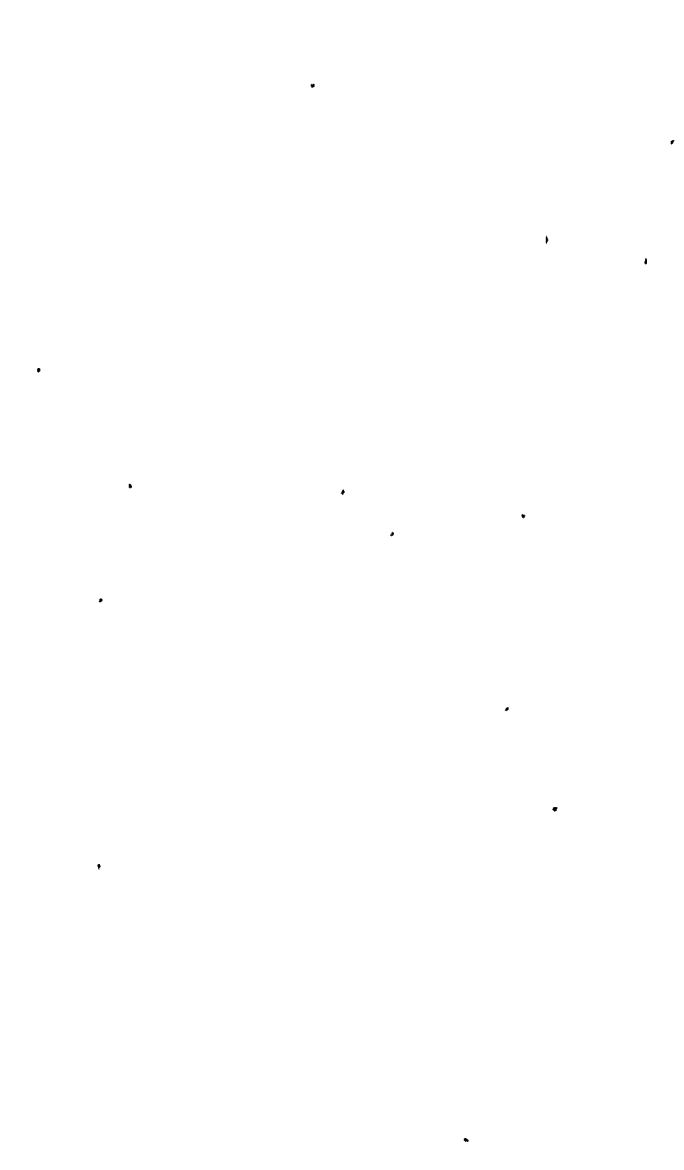
Rinse briefly in water, dehydrate, clear and mount.

*Preparation of CAF.* Melt 0.4 g. phenol, add 1 g. acid fuchsin (C.I. 692) and mix. Dissolve when cool in 10 c.c. 95 per cent. ethanol and make up to 100 c.c. with starch-dextrin solution prepared as follows. Grind 0.5 g. starch to a fine powder, then grind in 0.5 g. dextrin, add 100 c.c. water gradually with grinding, heat the mixture to about 80° C. and, when cool again, filter on to the phenol sludge solution.

*Notes on method.* The nuclear stain is removed in part by the subsequent treatment with iodine, and if it is desired to retain stronger nuclear detail it is advisable to stain with celestin blue prior to the hæmalum (Lendrum and McFarlane, 1940). The green stain is also gradually removed by the iodine, but stronger final green staining of the cytoplasm can be got by increasing the original time of staining with the fast green; the time in iodine should not be less than 2 minutes. The granules take up some of the green together with the subsequent fuchsin and are stained a blackish red that is very visible. The fuchsin goes also, although more slowly, into the collagen and basement membranes. The carbacid fuchsin has been found to give more vigorous staining than the usual acidified aqueous solution of acid fuchsin. The starch is used because it is reputed (and this has been roughly confirmed) even in low concentrations to increase the solubility of inorganic salts, dextrin because, as French (1929) reported, its presence certainly improves the Weigert type of elastica staining, and the phenol because it seems to have peculiar deviating effects on some acid dyes; a phenol eosin for example can, if sufficient phenol is used, be made to stain erythrocytes strongly and collagen hardly at all. This empirical recipe is worth the slight extra trouble of compounding.

### *The origin of the pale epithelium*

There has for long been dispute about the origin of this pale epithelium, the two main hypotheses being that it is either a sweat gland remnant (Lee *et al.*, 1933) or a secondary alteration of true mammary epithelium (Dawson, 1932; Geschickter, 1943). Tissues from the skin of the axilla have been examined





### PLATE XXXV

- FIG. 1.—Case 1234/39. This shows the darkly stained granule masses, mainly at the free end of the cells. In places the basement membrane shows the serrated appearance produced by a (partially) tangential cut of the basal so-called myo-epithelium. Ortho process. Stain H.FCF.CAF. Ilford 9.  $\times 120$ .
- FIG. 2.—Case 1234/39. Even with this lower contrast stain it is possible to recognise the large number of cells containing granules. The typical intracystic projections of the epithelium are well seen. Ortho process. Stain hæmalum-phloxin-tartrazine. Ilford 9.  $\times 120$ .
- FIG. 3.—Case 1234/39. The nature and disposition of the granules are well seen. In this field the granules are towards the free end but the characteristic cytoplasmic cap is visible in some of the cells. Ortho process. Stain hæmalum-phloxin-tartrazine. Ilford 9.  $\times 315$ .
- FIG. 4.—Case 1234/39. The granule-containing cells are here low cuboidal in type and show granules throughout the cytoplasm. Ortho process. Stain H.FCF.CAF. Ilford 9.  $\times 360$ .

PINK CELL CYSTOMA

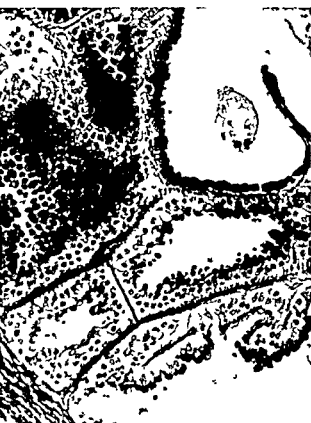


FIG 1



FIG. 2

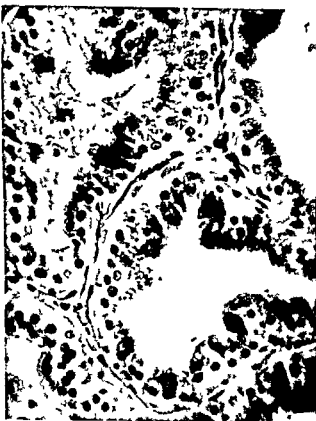


FIG 3

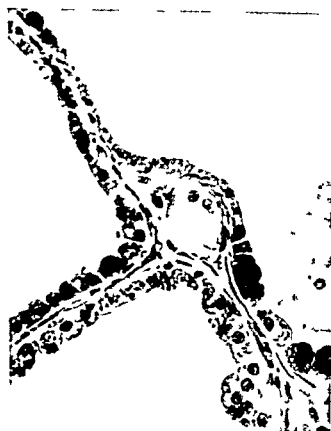


FIG 4



## PINK CELL CYSTOMA

FIG 5—Case 1234/39 This shows the concentration of granules in the supranuclear position, with the somewhat hyaline overlying domed cap. Ortho process. Stain H FCF CAF Ilford 9  $\times 375$

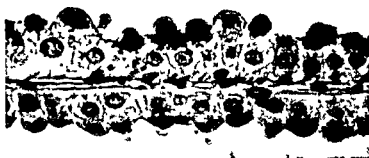


FIG 6—Case H 77 These larger cells show well the discrete nature of the granules. Toward one end is seen the bulbous swollen type of cell that suggests an imminent discharge of the granules. Half tone pan. Stain H FCF CAF. Ilford 2 and 4  $\times 375$



FIG 7—Case 927/44 Here the bulbous projection of certain cells is associated with a relative absence of granules. Ortho process. Stain H I Cl CAF Ilford 9  $\times 375$



FIG 8—Case 1234/39 The appearances here suggest a breakdown of the swollen domed cap and an accompanying lysis of the granules. No free granules have been seen in any specimen. Ortho process. Stain H FCF CAF Ilford 2 and 6  $\times 600$



by this new method and the findings certainly confirm the view expressed by Schultz that there is morphological identity between the cells of the apocrine glands in the axilla and the pale epithelium of the breast. Schultz gives coloured drawings (p. 38) of sections stained for fat and by the Turnbull blue method for iron, in which the apocrine glands are identical with his illustrations, from similarly treated sections, of pale epithelium in the breast (p. 91). My own observations include that of an area of apocrine-like glands found lying alongside typical sebaceous glands in a dermoid cyst of ovary; here also the new method gives a picture identical with that found in the breast and in the axillary apocrine glands.

General pathological principles suggest that the presence of clearly defined granules in an epithelial cell points to that cell's being metabolically active in a specialised capacity, and certainly not in a state of morbidity or stage of degeneration. This view was accepted for the Paneth cells found by us in a papilloma of gall bladder (Kerr and Lendrum, 1935-36); but as Magnus later observed (1937), the islets of intestinal epithelium to be found in the gastric mucosa should be considered, in virtue of their relationship to chronic irritative changes and despite their content of Paneth cells, as being areas of metaplasia. The pathologist, knowing how well defined can be the hyaline granules of renal tubular epithelium (Muir, 1941, fig. 411, p. 660) is not likely to forget that the presence of granules may be a manifestation of reabsorption. But from the observations of others (Schultz) and my own (see figs.) there seems little doubt that the granules in these breast cells are excretory, as indeed the Paneth cell granules are believed to be. If we accept Magnus's conclusions, which confirm the work of earlier German writers, it would seem not unreasonable to look on the occurrence of pale epithelium in the breast as being comparable to the occurrence of intestinal islets in the chronically irritated gastric mucosa. Thus in the stomach there occur islets of intestinal mucosa which, by their reported association with chronic irritative change, are considered as a metaplasia rather than an example of heterotopia, even though their epithelium has all the characters that we take to be indicative of the specialised nature of intestinal epithelium, namely goblet cells and the two granule-containing types—enterochromaffin and Paneth cells. Similarly in the breast the seat of chronic cystic disease, there is found an epithelium possessing all the appearances—cellular shapes, granule content and disposition of granules—which we recognise as being those of the specialised apocrine sweat glands. May we then regard this change in mammary epithelium as a metaplasia? Such a type of metaplasia could be considered as a misdirected regeneration, the epithelium of breast or stomach becoming dedifferentiated to the primitive sweat gland or primitive alimentary type, and in its subsequent regeneration acquiring the characteristics of another type of specialised epithelium which had originally developed from that primitive type. Thus gastric and intestinal epithelium both arise from primitive alimentary epithelium, and the rather more specialised gastric epithelium, if chronically irritated, may show misdirected regeneration to a fully formed small-intestinal type; while mammary epithelium and the epithelium of apocrine glands both arise from primitive sweat gland epithelium, and the rather more specialised mammary epithelium may after dedifferentiation show misdirected regeneration to a fully formed apocrine type.

#### Summary

A staining method is described which shows up distinctly the granules of the so-called pale or "pink" epithelium commonly found in cystic disease of the breast. The great similarity of this epithelium to that of apocrine sweat glands from the axilla and from a dermoid cyst of ovary suggests a comparison with the islets of intestinal epithelium found in gastric mucosa, and the occurrence

of pale epithelium is put forward as a form of metaplasia due to misdirected regeneration.

My thanks are due to Messrs W. Carson and W. Penny for their careful assistance in the preparation of slides and in the photography, and to the Rankin Fund for the cost of materials.

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### ON THE STAINING OF BASEMENT MEMBRANES

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(PLATE XXXVII)

By the use of a method originally devised for the staining of granules (Lendrum, 1945), it has been found possible to stain the basement membrane of the renal glomerulus with a new precision. The first application of this method to the kidney gave a sharp and specific delineation of the membrane but subsequent attempts failed until it was realised that the first sections had been affixed to the slides by Masson's gelatin method (Mallory, 1938). It was then discovered that the essential manoeuvre for success was the subjection of the section, still in wax, to the effect of hot formalin vapour; it is enough if the wax section affixed to the slide be kept in a closed dish, in which there is a layer of commercial formalin, at a temperature of 55-65° C. for about 2 hours. Staining is then carried out as detailed elsewhere (Lendrum).

The photographs are from sections stained with hæmalum as nuclear stain. If stronger nuclear detail is wished, then celestin blue staining should precede

## BASEMENT MEMBRANES



FIG 1—Ortho process Stain H & E CF CAF Filter Ilford 9 This shows the delicate membrane of the normal glomerulus in those loops that are patent The closed state of many of the loops is characteristic of the normal  $\times 315$



FIG 2—Ortho process Stain H & E CF CAF Filter Ilford 9 This reveals the generalised patency of the capillary loops and the appearance of thickening and rigidity of the membrane characteristic of eclampsia  $\times 315$



FIG 3—Ortho process Stain H & E CF CAF Filter Ilford 9 This shows the presence between the columns of hepatic cells and the basement membrane of the sinusoids of a pale staining finely granular material—a peri-trabecular oedema  $\times 490$





the hæmalum (*op. cit.*); this gives a general picture of the glomerular constituents which in clarity and definition is well above the average obtainable with Mallory's method or the Masson types of modification. Pre-treatment with formalin vapour failed to improve the staining of the membranes by the orange and blue method (Dunn, 1934). The present method has the further advantage over the orange and blue method that one can use a green screen for visual observation of the membranes; the photomicrographs were focussed with a green screen and then taken on ortho process plates through an Ilford filter 9. Fig. 1 is from a fairly normal adult kidney (age 52), and fig. 2 from a fatal case of eclampsia gravidarum. The section from which the latter was taken shows clearly the thickening of the membrane, its occasional fibrillated state and the stiffly rounded lumina described by Baird and Dunn (1933). Fig. 3 shows the separation by œdema fluid of the basement membrane from the trabeculae of hepatic cells in an œdematous congested liver from a case of intracapillary glomerulonephritis.

#### Summary

A method originally devised for the staining of granules has been found to stain basement membranes with great clarity if the wax section is first treated with hot formalin vapour.

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#### SIDEROCYTES IN HÆMOLYTIC DISEASES: A NEW INDEX OF SEVERITY AND PROGRESS

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Siderocytes are erythrocytes containing fragments of non-hæmatin iron which is stainable by appropriate methods. The cells were so named and brought into prominence by Grüneberg (1941 *a* and *b*) and were reported as occurring in certain pathological conditions in man by Doniach, Grüneberg and Pearson (1943). Non-hæmatin iron in erythrocytes had already been noted by Macallum (1895-96), Ehrlich and Lazarus (1898) and Proescher and Arkush (1927-28), though little attention seems to have been paid to these findings. Recently the concept of the siderocyte as an ageing erythrocyte has been introduced independently by Case (1943, 1945) and Granick (1943) and normal levels in man were established in a series of 279 subjects by Case, Ladan and Nutt (1945).

A new and delicate staining technique, using  $\alpha$ -dipyridyl and potassium thiocyanate, has also been elaborated (Case, 1944-45), though in these studies some of the preparations were stained by the technique of Macallum (1895-96) or Nishimura (1910). Films must be fixed, stained and counted within three days of being made, or an artefact rise in siderocyte level occurs (A. Ireland, personal communication). Increased accuracy for low counts and a saving

of time in high counts may be obtained with the sampling method of Haldane (1945).

A study of the physiological properties of the siderocyte (Case, 1943, 1945) showed that the siderotic granules are extruded, giving an increase of plasma iron, that the cell after extrusion of the granules is susceptible of phagocytosis and that the onset of a high siderocyte level is accompanied by the appearance of urinary siderotic granules as described by Rous (1918) in untreated pernicious anaemia and in hæmochromatosis. Siderocytes should therefore be found in diseases characterised by a high rate of erythrocyte destruction and urinary siderotic granules should also make their appearance in association with a high siderocyte level. The following studies show that such is indeed the case (table I) and suggest that the siderocyte count can be a sensitive and useful guide in assessing the severity and progress of hemolytic conditions.

TABLE I

*Siderocyte levels in health and in various hemolytic and other conditions*

	No. of cases examined	Percentage of siderocytes
Normal	279	0.5 - 0.8
Hypochromic microcytic anaemia	60	1 - 3
Hæmochromatosis	4	3 - 7
Bacterial toxæmias	3	6 - 10
Severe burns	5	3 - 10
Industrial solvent poisoning	3	9 - 10
Untreated pernicious anaemia	3	8 - 14
Clinical lead poisoning	4	10 - 30
Sickle-cell anaemia	1	40
Acholic jaundice	5	20 - 100

Thus it is seen that with the exception of hæmochromatosis, which is not thought to be a hemolytic condition, the siderocyte levels follow the severity of the hemolytic process reasonably closely.

Further information is obtained by following the day to day variation in the siderocyte level. Three cases are given in detail (table II).

TABLE II

*The siderocyte and reticulocyte response in three cases of pernicious anaemia under treatment with liver extract*

Day of therapy	E. W., female, aet. 50						D. C., male, aet. 71						R. R., male, aet. 55					
	S	R	Hb	RBC	R/S	US	S	R	Hb	RBC	R/S	US	S	R	Hb	RBC	R/S	US
Before	13.0	2.0	58	1.71	0.5	++	8.0	2.0	56	2.3	0.25	+++	14.0	2.0	42	2.0	0.14	+
1	12.0	1.9	56	1.72	0.16	++	7.8	2.0	56	2.2	0.25	+++	13.0	2.0	40	2.0	0.15	++
2	10.0	2.0	55	1.68	0.2	++	7.0	2.1	54	2.3	0.30	+++	12.0	2.1	43	1.8	0.13	++
3	8.0	2.2	58	1.50	0.25	++	6.5	2.3	57	2.2	0.35	+++	11.5	2.0	40	1.9	0.17	++
4	6.5	15.0	65	1.49	2.3	++	6.0	7.8	52	2.1	1.3	++	10.0	2.0	40	2.0	0.5	+
5	5.8	10.0	66	1.50	1.7	++	5.0	7.0	56	2.2	1.4	++	12.0	12.0	55	2.5	1.0	+
6	2.8	18.0	68	2.2	6.4	++	4.0	10.0	42	2.1	2.5	+	8.0	9.0	60	2.8	4.6	+
7	2.1	12.0	68	2.4	5.7	++	3.6	8.0	58	2.6	2.2	+	5.5	18.0	62	2.9	3.3	+
8	2.0	18.0	70	2.8	9.0	++	2.5	10.0	63	2.8	4.0	tr.	9.6	12.0	64	3.0	10.3	+
9	1.9	18.0	69	2.8	9.4	+	1.3	15.0	64	3.1	11.5	tr.	1.9	20.0	65	3.1	4.8	+
10	1.6	10.0	70	3.0	6.2	+	1.2	15.0	67	3.1	12.2	—	2.1	10.0	63	3.1	10.2	+
11	1.0	20.0	68	3.0	20.0	tr.	...	...	...	...	...	...	1.9	20.0	65	3.2	10.7	tr.
12	1.0	10.0	70	3.0	10.0	tr.	...	...	...	...	...	...	1.2	20.0	68	3.2	...	...

S = siderocytes per cent.

Hb. 100 per cent. = 14.8 g. per 100 g.

R/S = hemodynamic index.

R = reticulocytes per cent.

R.B.C. per million.

US = urinary siderosis.

Therapy : 4 ml. liver extract intramuscularly on alternate days.

The first reaction to liver extract therapy, occurring well before the reticulocyte response, is in the siderocyte level, whilst the reticulocyte siderocyte ratio, or, as suggested to me by Gruneberg, "hæmodynamic index", which normally lies between 2 and 5, is at first low and then becomes high. It is suggested that such an index might be of use in following the progress of hæmolytic processes. The urinary siderosis becomes less marked as the siderocyte level falls.

Phenylhydrazine, in both the normal subject and in polycythæmia vera, increases the siderocyte count in a manner directly proportional to the fall in the erythrocyte count and also causes the appearance of urinary siderotic granules. Splenectomy, in the one case of acholuric jaundice followed, caused a fall of both siderocytes and reticulocytes to normal levels, whilst a marked improvement in the clinical condition took place. Benzene, trichlorethylene (commercial) and lead all cause siderocytosis.

Thus it would appear that the siderocyte level may be of use as a diagnostic and prognostic guide in clinical medicine, and the "hæmodynamic index" might be a convenient method of correlating this level with the reticulocyte level.

In pernicious anæmia the siderocytes are not stainable with prussian blue unless unmasked by the Nishimura technique, and as yet no information is available as to the efficiency of the  $\alpha$ 'dipyridyl thiocyanate technique in this disease.

It is a pleasure to acknowledge my indebtedness to Mr C. Lees, Mr A. Ireland and Miss V. Ladan for their careful work in the preparation and examination of material, and I am grateful to the honorary staff of the Birmingham United Hospital for allowing me to study the clinical material.

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## BOOKS RECEIVED

### Immuno-catalysis

By M G SEVAG 1945 Springfield Ill and Baltimore, Md Charles C Thomas (Distributors in the British Isles, Baillière, Tindall and Cox) Pp xv and 272 \$4 50 (25s)

In spite of the impression of permanence which the appearance and price of this book convey it is to be regarded as essentially an essay<sup>1</sup> The thesis which it presents is briefly that antigens act catalytically in evoking antibody response No rigid proof is attempted, but a mass of biochemical, biophysical and immunological data not ordinarily found in a single volume is set out and interwoven with the aim of establishing a close analogy between antigens and chemical catalysts Enzymes, having the specificity of the antigen while behaving stoichiometrically according to the laws of catalysis, are invoked as the essential link to bridge the gap between the two Since they act in the test tube as well as in the organism they help one to swallow a most indigestible feature of the analogy, namely the projecting of a strict physical law on to a peculiarly vital process

The analogy is not new but it is presented in a setting of recent discoveries It is usually plausible and sometimes striking but leaves the reviewer with a sense of energy misspent, since little that is of practical value emerges It may be useful as a skeleton on which to hang a mass of biological and chemical data, but it fails notably to conjure up to the mind new and exciting experiments and this seems to the reviewer a more important test of an analogy than its ability to regiment data

Some of the information is unfortunately presented with a lack of critical insight For instance, in emphasising an identity of fundamental molecular structure in normal and immune globulins of the same serum an altogether exaggerated sensitivity is attributed to sedimentation and electrophoretic criteria of individuality This leads often to deductions which a conservative mind is not willing to accept, for example, that since antibodies and normal globulins are practically indistinguishable the synthetic process for both is the same

This is a book for the specialist by whom it will be read critically The reader who is not familiar with recent immunology and protein chemistry will find it tedious and confusing

### Root disease fungi

By S D GARRETT 1944 Waltham Mass, The Chronica Botanica Co London, William Dawson & Sons, Ltd Pp xiv and 177, 9 text figs and 1 plate \$4 50

It was not until the middle of the 19th century that certain fungi were recognised to be the cause and not the result of disease, and for many years after this the attention of mycologists was devoted almost exclusively to the aetiology of fungus diseases and to methods of killing or controlling the parasites by direct means Since the beginning of this century, however, the emphasis has gradually been shifting, more and more attention has been given to the all important effects of soil and weather conditions on



in 1914-18, in the advance of pathological knowledge in several directions". The authors have endeavoured to include the salient points of this newer knowledge, especially in respect of the crush syndrome, hepatitis, shock, wounds and infections, and the problems arising from blood incompatibility. The text as a whole has been reviewed and partly rewritten, and new chapters have been added. In all, the text has been expanded by some 20 pages.

This well written and beautifully illustrated text-book has proved to be, as we had expected, deservedly popular with students and is likely to remain so in its new edition. It has a distinct pull over other present-day manuals of pathology in that it deals very fully with the bacteriological and immunological aspects of infective diseases. The chapter on diseases due to animal parasites (31 pages) is a useful and welcome addition. It includes short but pithy accounts of malaria and the principal worm and fluke infestations of man. The drawing of the scolex of *Taenia saginata* (in fig. 303), however, looks like a slightly enlarged scolex of *T. solium* with the rostellum and its hooklets amputated, while the scolex of *T. solium* alongside is shown with only a single row of hooklets.

There are still a few subjects, not perhaps of major importance, which we think might well receive notice in future editions—adamantinoma, argentaffine tumours, benign calcified epithelioma, chordoma, mucocele of the appendix as a cause of pseudomyxoma peritonei, melanosis coli and pancreatic islet tumours. Most of these conditions are not rare and some are of considerable practical importance. We think their inclusion would still further enhance the merits of this highly meritorious work.

#### A text-book of surgical pathology

By C. F. W. ILLINGWORTH and B. M. DICK. Fifth edition, 1945. London: J. & A. Churchill. Pp. viii and 728; 306 text figs. 42s.

Apart from a preliminary outburst against the writing of surgical pathologies, and a few constructive criticisms of succeeding editions, we have had nothing but good to say of this now well established manual.

"In this new edition a number of alterations and additions have been made without adding to the size of the volume. The sections on surgical shock and burns have been rewritten and incorporated in a new chapter under the title of Constitutional Effects of Injury. The section dealing with anaerobic wound infections has been revised to bring it into line with the experiences gained in the present war. A number of new subjects have been included, chief of which are the crush syndrome, sarcoidosis of Boeck, solitary plasmacytoma of bone, adenolymphoma of the salivary glands, argentaffine tumours of the intestine, and interstitial-cell tumour of the testis". Thus the authors, in their preface to the fifth edition. It only remains to say that these additions to the text are as thoroughly trustworthy as the rest of the book, which remains an admirable guide to the pathology of diseases commonly classified as "surgical".

#### Handbook of practical bacteriology

By T. J. MACKIE and J. E. MCCARTNEY. Seventh edition, 1945. Edinburgh: E. and S. Livingstone. Pp. viii and 720. 17s. 6d.

In the latest edition of this useful little book new material has been for the most part incorporated in the appendix, which now consists of 43 pages instead of the 7 of the sixth edition. This increase, with a few extra pages in the virus section, accounts for the overall increase of 44 pages in length.



Throughout the greater part of the book there are few alterations, but some small additions have been made to bring the material up to date, as in the improved section on the diagnosis of diphtheria (p. 386) and on the phage typing of *Bact. paratyphosum B* (p. 435), the rickettsial agglutination test in the diagnosis of typhus fever (p. 564), the relationship of psittacosis virus to others of the group (p. 629) and atypical pneumonia (p. 635).

The new matter in the appendix includes sections on penicillin, fluorescence microscopy, the cultivation of anaerobes and the bacteriology of infected wounds, and a description of newer technical methods and media. The quality of the paper is not quite as good as in the previous edition.

#### **Studies on immunisation : second series**

By Sir ALMROTH E. WRIGHT. London : William Heinemann (Medical Books) Ltd. Pp. vii and 256 ; 17 text figs. and 1 plate (6 figs.) in colour. 25s.

This volume, like its predecessor, serves up old material in elegant form. Among the subjects described are the value of prophylactic inoculation against pneumonia, the pathology and treatment of tuberculosis and a critical discussion on theories of immunity. Some of the nine articles and four appendices were written more than thirty years ago. As before, tables and figures fill pages of valuable paper when single sentences would often suffice.

# PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

23rd and 24th March 1945

The sixty-eighth meeting of the Society was held in the Department of Pathology, St Thomas's Hospital Medical School, London, S.E. 1, on Friday and Saturday, 23rd and 24th March 1945.

## Communications and demonstrations

Those marked with an asterisk are abstracted below

- J. G. THOMSON. Death from bronchial asthma with asthmatic reaction in an ovarian teratoma.
- J. W. ALLEN, G. R. CAMERON, R. F. G. COLES and J. P. RUTLAND. The effects of applying pressure to experimental thermal burns.
- DOROTHY S. RUSSELL and J. PENNYBOCKER. Irradiation necrosis of the human brain.
- R. F. OOLVIE. Growth in relation to the diabetogenic and pancreotropic actions of anterior pituitary extract.
- L. HOYLE and J. W. ORR. The histogenesis of experimental pneumonia in mice.
- D. M. PRYCE. Abnormal arteries in the causation of diaphragmatic hernia.
- R. A. M. CASE. (1) Siderocytes in hemolytic conditions. A new index of severity and progress. (2) An analysis of punctate basophil levels and siderocyte levels in lead workers and in lead poisoning.
- \*G. PORJAK. Absorption of cholesterol from a watery suspension and its effect on plasma lipids.
- \*E. J. KING, NANCY ROGERS and MARGARET GILCHRIST. Experiments with right and left quartz, and attempts to prevent silicosis with aluminium.
- A. GLUCKSMANN. The histogenesis of benzpyrene-induced epidermal tumours in the mouse.
- R. J. LUDFORD. Colchicine in the experimental chemotherapy of cancer.
- L. DMOCHOWSKI. Induction of breast cancer in mice by the mammary tumour agent.
- \*E. M. BRIEGER. Observations on the species pathogenicity of the tubercle bacillus in intra-amniotic infection.
- R. J. V. PULVERTAFT. Liesegang rings in blood-agar shake cultures.
- L. P. GARROD. Penicillin sensitivity tests.
- K. E. COOPER and D. WOODMAN. The diffusion of antiseptics through agar gels.
- D. G. EVANS. The treatment with antitoxin of experimental gas gangrene in guinea-pigs.
- G. S. WILSON and J. D. ATKINSON. Typing of staphylococci by the bacteriophage method.
- R. E. O. WILLIAMS. Further observations on the carriage of *Staph. aureus* in man.
- J. H. DIBLE. The peripheral arteries in a case of symmetrical gangrene of the extremities.
- E. M. BRIEGER, H. B. FELL and C. ROBINOW. Warm-stage and cytological observations on the early development of the avian tubercle bacillus.
- R. E. O. WILLIAMS and G. J. HARPER. A sheep-blood agar plate for the demonstration of staphylococcal  $\alpha$ -haemolysin.

\*L. J. EDWARDS. Two cases of atypical amyloidosis.

E. J. KING and MARGARET GILCHRIST. Field methods for mepacrine.

D. M. PRYCE. Four cases of intralobular sequestered lung.

EMMY KLIENEBERGER-NOBEL. Spore formation in bacteria and actinomycetes.  
J. N. J. HARTLEY and J. S. FAULDS. (1) Multiple carcinoid tumours of the small intestine in siblings. (2) Calcification of lung in a case of diffuse plasmacytoma of the spine.

IAN RANNIE. Focal pituitary necrosis unrelated to pregnancy.

R. M. HAINES. Pancreatic islet-cell tumour.

L. R. WOODHOUSE PRICE. (1) Sarcoma of prostate. (2) Endosteal fibrosarcoma. (3) Xeroderma pigmentosum. (4) Periosteal chondrosarcoma. (5) Malignant synovioma of knee joint with xanthomatous reaction. (6) Adenolymphoma of parotid.

P. BRUCE WHITE. A method for the assay of penicillin.

### Abstracts

612 . 322 . 73 : 547 . 922

## ABSORPTION OF CHOLESTEROL FROM A WATERY SUSPENSION AND ITS EFFECTS ON PLASMA LIPIDS

G. PORJÁK, Beit Memorial Fellow for Medical Research

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That cholesterol is not absorbed to any appreciable extent from the alimentary tract unless it is administered dissolved in fats is true only for crystalline cholesterol. Dömösi and Egyed (1939) showed that amorphous cholesterol suspended in water is readily absorbed from the alimentary tract of rabbits. When crystalline cholesterol dissolved in hot glacial acetic acid is poured into a large volume of cold water, it is precipitated in an amorphous form. The acid can be removed by repeated washing with water on a filter paper. The findings of Dömösi and Egyed were confirmed. The absorption of cholesterol is a delayed process, the earliest changes in the plasma lipids of rabbits being only observable 24 hours after the administration of 1 g. of cholesterol. After prolonged cholesterol feeding very high degrees of hyperlipæmia were found, the total plasma cholesterol values having reached 2000-3000 mg./100 ml. The ratio, ester/free cholesterol, is usually but not consistently increased. Together with the rise in plasma cholesterol values there was a rise in non-phospholipid fatty acids and in phospholipids as well. It was found that the plasma-free cholesterol values, normal and pathological, bore a quantitative relationship to non-phospholipid fatty acids on the one hand and to phospholipid values on the other. The former relationship can be expressed by the equation  $y = 11.508x^{0.752}$ , and the latter by the equation  $y = 14.431x^{0.653}$  with an error of  $\pm 10$  per cent. ( $x$  = mg. of free cholesterol and  $y$  = mg. of non-phospholipid fatty acids, or phospholipids per 100 ml. of plasma). The equations are applicable also to species other than the rabbit, including man.

It is concluded from the data obtained that the level of free cholesterol in the blood plasma regulates fat metabolism by determining the rate of mobilisation of fatty acids from the depots and the rate of phospholipid synthesis.

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## ATTEMPTS TO PREVENT SILICOSIS WITH ALUMINIUM

E. J. KING, NANCY ROGERS and MARGARET GILCHRIST

*. British Postgraduate Medical School*

Following on the unsuccessful attempts of Belt and King (1943) to prevent experimental silicosis with metallic aluminium, we obtained a specimen of specially prepared aluminium dust from the Canadian workers (Denny, Robson and Irwin, 1939; Crombie, Blaisdell and MacPherson, 1944). This was intimately mixed with finely ground Brazilian quartz (obtained by courtesy of Dr E. L. Middleton) by the special technique described for us by Dr D. A. Irwin.

Four hundred mg. doses of the mixture (98 per cent. quartz, 2 per cent. aluminium) suspended in 4 ml. of saline, were introduced into the lungs of 12 unanæsthetised rabbits through a soft rubber catheter inserted into the trachea as far as the bifurcation. Six control rabbits received quartz without the aluminium. Both groups of animals tolerated the dose well; they were killed (or died) at monthly intervals up to 1 year and the lungs examined.

*Results with quartz.* The changes produced in the lungs were characteristic of the experimental silicotic lesion. They consisted of intra-alveolar nodular foci of macrophages containing quartz crystals (koniophages). The older lesions showed well developed hyaline change, with reticulin hyperplasia advancing in many areas to a reticulin fibrosis and some collagen formation (cf. Belt and King, 1945). A number of the foci contained foreign-body giant cells indicating that some of the particles were too large to be dealt with by macrophages.

*Results with quartz plus aluminium.* Silicosis was not prevented, although the lesions were less advanced than in the lungs of the quartz animals. The lesions were similar to those of the controls, but the sections cut from the blocks containing the most aurine-positive material (i.e. containing most aluminium) showed a greater tendency to confluence and a lesser tendency to hyaline change with reticulin fibrosis. Collagen formation was less marked than in the controls. These changes were, however, slight, and possibly without significance.

In all sections, aurine-staining material was exceedingly scarce. Evidently the 2 per cent. of aluminium in the original mixture had been greatly reduced by the time the animals were killed. Absorption of the aluminium had obviously been preferential, since large patches of the crystalline quartz were clearly visible in the incinerated sections.

It appears probable that if experimental silicosis is to be prevented in animals treated with powdered quartz by the Kettle intra-tracheal technique, much more than 2 per cent. of aluminium must be intimately mixed with the quartz or the aluminium in the lungs must be repeatedly renewed. Experiments directed to these ends are in progress.

Our thanks are due to the Medical Research Council for an expenses grant from which part of the cost of this investigation was defrayed.

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618.346—002.5:576.8.095.57

OBSERVATIONS ON THE SPECIES PATHOGENICITY OF  
THE TUBERCLE BACILLUS IN INTRA-AMNIOTIC INFECTION

E. M. BRIEGER

*From Papworth Village Settlement*

The mode of reproduction of avian and human tubercle bacilli was studied after each had been injected into the amniotic fluid of chick and guinea-pig embryos, the purpose being to study the behaviour of the bacilli in the organism of susceptible and resistant hosts. It was shown that the guinea-pig was most susceptible to this kind of infection by human tubercle bacilli while the avian produced neither microscopical nor macroscopical evidence of lesions. In the chick embryo the avian bacilli produced extensive lesions in the chorio-allantoic membrane, with foci also in the tissues of the embryo, while human bacilli only produced a few small foci in the chorio-allantoic membrane. Intra-amniotic injection of the guinea-pig with human bacilli and of the chick embryo with avian bacilli was therefore considered the suitable method of demonstrating the response of a susceptible host to infection, while intra-amniotic injection of the guinea-pig with avian bacilli and of the chick embryo with the human strain was used to show the response of a resistant host. In these experiments 50 fertile eggs were injected with human and avian bacilli respectively, while the guinea-pig experiments were carried out on 4 pregnant guinea-pigs, 2 being injected with avian and 2 with human bacilli. The following results were obtained.

Amniotic fluid withdrawn 3-5 days after inoculation (the embryo being still alive) contained only a small number of tubercle bacilli—there was no sign of marked multiplication. If drops of the infected fluid were transferred to Loewenstein's medium at this stage, colonies appeared in the normal time, irrespective of whether the fluid was that of a susceptible or resistant host, showing that the viability of the bacilli was not impaired. Avian strains recovered from the amniotic fluid of guinea-pigs, however, had completely lost their pathogenicity.

If after withdrawal the amniotic fluid was incubated *in vitro*, multiplication began, but was very different in fluid from susceptible and non-susceptible animals. In fluid from a susceptible host growth started immediately and proceeded quickly, the bacilli developing as in embryo extracts. (This was demonstrated at the meeting of the Society by Brieger, Fell and Robinow.) In the fluid from non-susceptible hosts, on the other hand, growth started slowly, remained poor and was greatly modified as compared with the normal development.

A characteristic feature of the preparations obtained after withdrawal was the presence of peculiar bodies in the amniotic fluid of susceptible and non-susceptible hosts alike, and in both chick and guinea-pig fluid. These bodies were notably predominant over the bacillary rods. They seemed to be organised structures which underwent developmental changes and consisted at the time of withdrawal of a mass of granules intermingled with some oddly shaped bacilli. The amorphous background stained dark blue with Gram and pinkish violet with Giemsa.

The nature of these structures is still obscure and the question must remain open whether the bacilli which develop when the infected amniotic fluid is withdrawn and incubated *in vitro* are derived from the bacilli of the inoculum or from the granular material described above. The paucity of bacilli in the amniotic fluid immediately after its withdrawal and the presence in abundance of the granular material just described suggest the latter possibility. Further work is in progress.

616—003 . 821

## TWO CASES OF ATYPICAL AMYLOIDOSIS

J. L. EDWARDS

*From the County Laboratory, Stafford*

## Case I

*Amyloid deposits in tongue, cervical lymph nodes and peritoneum:  
giant cell reaction to amyloid material*

*Clinical history* (Dr J. M. Cowan). Female, aet. 58, married, had been under treatment for severe rheumatoid arthritis and anæmia which did not respond to iron or liver. Masses resembling faecoliths were palpated in the abdomen. A barium enema disclosed no bowel defect. No complaint of tongue. Vomiting and abdominal pain appeared and the patient began to look ill. She now complained of tenderness of the tongue but there was no complaint of its size. On examination several hard white plaques were seen on the tongue. Liver and spleen not enlarged. No history of suppuration. Sedimentation rate always rapid. Diarrhoea was now present and the patient died from exhaustion.

*Post-mortem findings* (24 hours after death). Tongue as described above. Fixed bilateral masses, about  $1 \times \frac{1}{2}$  inch in diameter, were present in the submaxillary region. Liver, spleen and kidneys appeared normal. There was no evidence of suppuration, prolonged or otherwise. The bowel appeared normal. The anterior parietal peritoneum exhibited uniformly distributed roughly spherical masses  $\frac{1}{2}$  1 inch in diameter.

*Histology*. Amyloid deposits with irregular staining reactions to Congo red and iodine are present in the tongue and submaxillary lymph nodes and in the peritoneal "tumours". Most of the deposits stain with Mallory and methyl violet. Foreign body giant cells, apparently ingesting amyloid material, are present, especially in the submaxillary lymph glands.

*Commentary*. The distribution of the amyloid deposits and their staining reactions are very much as described, for example, by Barnard *et al.* (1938). Giant cell reaction to amyloid material has been described by Reimann *et al.* (1935) and described and illustrated by Wohlwill (1942).

## Case II

*Amyloid deposit in ureter causing hydronephrosis*

*Clinical history* (Mr L. E. Jones). Male, aet. 35, complained of right renal colic. Three attacks accompanied by hæmaturia occurred during one year. There were no other symptoms. He had contracted malaria while on service abroad. A sister had nephrectomy for tuberculosis eight years ago. The patient is well built and of healthy appearance, with a tendency to obesity. Tongue normal, no swellings palpable in head, neck or abdomen. Rectal examination negative. Wassermann negative. Blood pressure 130/70.

*Urine*. Specific gravity 1018. Trace of albumin. No reducing substance or acetone bodies. A few polymorph leucocytes and many red cells present, no casts. On culture, *B. proteus* and *B. coli* isolated.

Blood urea 54 mg. per 100 c.c. Uroselectan revealed good excretion on both sides. There was early right hydronephrosis, with dislocation of the upper end of the ureter opposite L 3. A funnel shaped constriction of the ureter was visible. Cystoscopic examination revealed nothing abnormal.

*Urea clearance test*

Time	Amount of urine received	Specific gravity	Urea (per cent.)
9.0 a.m.	56 c.c.	1025	2.4
10.0 "	62 "	1024	2.4
11.0 "	74 "	1021	2.5
12.0 noon	80 "	1024	2.5

*Operation* (Mr Jones). The right kidney was exposed and felt normal. About the brim of the pelvis the ureter was thickened and the thickening ran down irregularly to one inch above its entrance into the bladder. The kidney and diseased ureter were removed, the patient making an uneventful recovery. The kidney appeared normal to the naked eye, but the ureter was thickened and hard. On section its wall appeared translucent and grey, with a faint yellow tinge.

*Histology.* Hyaline deposits, some of which stain pink with methyl violet, are seen in the ureteric wall immediately subjacent to the epithelium and also in part replacing the muscle. A few plasma cells, polymorphs and collections of lymphocytes are present. The deposits do not stain characteristically with iodine, even after 24 hours; they stain blue with Mallory and, patchily, with Congo red. The kidney contains a small subscapular deposit near the ureteric termination.

*Commentary.* Apart from the three attacks of renal colic, with hæmaturia, and malaria, the patient had always been healthy and nephrectomy was followed by an uneventful recovery. The few polymorphs seen in the urine hardly justify the title of "suppuration". The case is unique so far as the writer has been able to discover.

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576 . 8 . 095 . 5 : 576 . 851 . 42 (*Br. bronchiseptica*)

## THE INFLUENCE OF SUBSTRATE ON THE VARIATION OF *BR. BRONCHISEPTICA*

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WHILE investigating the metabolism of *Br. bronchiseptica* in relation to the production of toxin it was found that normal strains grew on Koser's medium and produced toxin from this relatively simple substrate. After a few subcultures on Koser's medium a stable variant appeared which, among other differences, produced very much less toxin than the normal strain. Attention was turned therefore to the relation between substrate and variation and it is essentially with this question that the present paper deals. The concentration of Cl and other monovalent ions in the medium has been shown to have a marked effect in causing or suppressing the appearance of the variant and some compounds providing the energy source have had a similar effect.

### *Methods*

All strains were freshly isolated and preserved in that condition by drying from the frozen state *in vacuo*. This process is referred to throughout the paper simply as "drying". Strains NGP 1-7 were from the lungs of normal guinea pigs, strains D 1 and D 4 were from a dog, and strain R 1 was from a rabbit's lung.

Dried cultures when plated on Bordet Gengou medium (as modified by Gardner and Leshe, 1932) yielded only one form of colony, the normal ("N") colony for this organism. After incubation for 48 hours separate colonies were surrounded by a pronounced zone of haemolysis. The same appearance was obtained when the amount of blood in the medium was reduced from 50 to 25 per cent. but not with less, and it also occurred on a simple medium made by dissolving 4 g. of agar in 75 ml. of 0.85 per cent. solution of NaCl and



adding to it at 50° C. 25 ml. of oxalated horse blood. This medium, referred to as blood-saline agar, was used as a routine for detecting variant ("V") colonies which were not easily differentiated from N colonies except on media containing a large percentage of blood.

The formula of Koser's medium was:—NaCl (A.R.) 5 g.,  $\text{NH}_4\text{H}_2\text{PO}_4$  1 g.,  $\text{K}_2\text{HPO}_4$  (anhydrous) 1 g.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (A.R.) 0.2 g., sod. citrate (A.R.) 2.77 g., distilled water 1 litre. It was sterilised at 15 lb. pressure for 15 minutes. Koser's agar was made by solidifying Koser's medium with 2.5 per cent. of shredded agar which had been washed until no more salts could be demonstrated in the wash water.

Chemically clean hard glass tubes were used throughout. All cultures were incubated aerobically at 37° C. unless otherwise stated.

The method used for the production and titration of toxic extracts was that of Evans and Maitland (1937), who extracted toxin from bacteria which had been killed by freezing and thawing and ground in an agate mortar. The extract was titrated by intradermal inoculation into the shaved back of a rabbit. Toxin could also be titrated by intradermal injection of washed living organisms. Bacteria, dried after washing in saline and distilled water, were suspended in distilled water (2 ml./mg. dry weight) and 0.2 ml. of serial dilutions inoculated, the end-point, as in extract titrations, being the highest dilution causing a definite necrotic spot. This method was convenient for indicating large differences in toxicity and gave parallel results to titration of extract which, being more accurate, was used to confirm the preliminary titrations of washed living organisms.

## EXPERIMENTAL

### *Production of the variant and comparison with the normal form of Br. bronchiseptica*

When *Br. bronchiseptica* was repeatedly transferred on slopes of Bordet-Gengou medium and at intervals plated out to obtain separate colonies, after about 12 subcultures variant colonies appeared and during a few more transfers they rapidly predominated. Subcultures from any one of these colonies gave a stable variant. If, however, a normal colony was always selected for transfer on plates the culture remained in the normal state for at least 50 subcultures.

The variant was produced similarly by growth in Koser's medium. Starting from a single N colony, subcultures were made every two days in tubes of liquid Koser's medium, with periodic plating on blood-saline agar. The variant appeared usually within 10-12 subcultures and ten strains were alike in this respect. No intermediate forms were observed and cultures made from V colonies gave rise to V colonies only. V cultures have never reverted to the N type; even after several hundred subcultures they continued to show the same properties as when first separated from the parent strain.

In one experiment Koser's medium was treated with activated charcoal before use with the object of removing traces of organic impurities which might have been present, but this made no difference to the results. The change from N to V forms occurred as readily during subculture on Koser's agar slopes as in liquid Koser's medium.

The variant did not appear when N strains were subcultured in

Koser's medium at 22° C., strains NGP 1, NGP 2, D 1 and R 1 having been tested in this respect through 100 subcultures. Neither did it appear on prolonged incubation in Koser's medium.

*Morphology.* Both N and V forms were Gram-negative, motile cocco-bacilli with peritrichate flagella. No abnormal forms were ever observed. By the use of a modified Muir's stain both forms were shown to be capsulated.

*Colony formation.* N and V cultures were not easily differentiated by colony appearance on nutrient, yeastrel or 5 per cent. blood agar or on any medium not containing large amounts of blood. After 48 hours N colonies on blood-saline agar were domed, had a glistening milky appearance and were surrounded by a pronounced zone of hæmolysis. V colonies were much larger, flat, oval and translucent, with no hæmolytic zone around separate colonies, though there was evidence of hæmolysis around areas of confluent growth.

*Biochemical tests.* N and V forms were indistinguishable by the usual biochemical tests.

*Agglutination.* Anti-N serum agglutinated both N and V organisms to titre, as did anti-V serum. Anti-N serum absorbed with N had all agglutinins removed; absorbed with V the serum failed to agglutinate V but still agglutinated N approximately to titre. Absorption of anti-V serum with either V or N removed all agglutinins. These results could be explained by assuming that N cultures possess two antigens, N and V, and that V cultures possess only V.

*Toxicity.* Comparison of the M.R.D. of N and V cultures was made on the same rabbit and each pair of strains to be tested was grown on the same batch of medium. The N form of strains NGP 1 and 2 was 8-30 times more toxic than the V form, irrespective of the medium in which they had been grown, of the number of times the V form had been subcultured and whether living organisms or extracts were tested.

Evans (1940) prepared *H. pertussis* antitoxic serum and showed that it neutralised the toxic extract of normal strains of *Br. bronchiseptica*. Using antitoxic serum supplied by him and following his technique, both N and V toxic extracts were shown to be neutralised.

*Hæmolysin production.* On blood-saline agar plates it appeared that the V strain produced much less hæmolysin than the N strain. Attempts to estimate this accurately were not successful, nor could hæmolysin production be correlated with toxicity except that both, though much reduced in the V form, were present. Potent N toxic extracts did not hæmolyse a saline suspension of horse blood cells, using Henry's technique (1922).

*Pathogenicity for guinea-pigs.* N and V cultures of strain NGP 2 were grown for 48 hours on Koser's agar. After washing and drying, weighed amounts suspended in saline were injected intraperitoneally into guinea-pigs, three animals per dose being used. The dosage was calculated as mg. dry weight per 100 g. body weight and in assessing

potency the time till death was also considered. The results indicated an M.L.D. of approximately 0.06 mg./100 g. body weight for the N form and approximately 2.0 mg./100 g. for the V form (table I). Both N and V cultures produced post-mortem changes characteristic of *Br. bronchiseptica* as described by Evans and Maitland (1937, 1939), and the corresponding organism to the one injected was recovered in pure culture from the peritoneal exudate and heart blood in every case.

When toxic extracts were employed, intraperitoneal injection of 1 ml. of a 1 : 50 dilution of N extract per 100 g. body weight killed 2/2 guinea-pigs, whereas a dose of 1 ml. of a 1 : 5 dilution of V extract was required to produce this effect (table I). The post-mortem appearances resulting from N or V extracts were similar to those caused by living organisms.

TABLE I

*Pathogenicity of N and V cultures and extracts of strain NGP 2 injected intraperitoneally in guinea-pigs*

	Dosage per 100 g. body weight	Deaths	Time till death in days
<b>Washed living organisms</b>			
Normal . . . {	0.5 mg.	3/3	2, 2, 3
	0.25 "	3/3	3, 3, 3
	0.125 "	3/3	5, 5, 3
	0.06 "	3/3	5, 5, 5
	0.03 "	0/3	...
Variant . . . {	2.0 "	3/3	3, 1, 1
	1.0 "	1/3	1
	0.5 "	1/3	1
<b>Extracts</b>			
Normal . . . {	1 ml. 1 : 30	2/2	2, 2
	1 ml. 1 : 50	2/2	7, 9
	1 ml. 1 : 100	1/2	8
Variant . . . {	1 ml. 1 : 5	2/2	1, 1
	1 ml. 1 : 10	0/2	...

These experiments indicated that pathogenicity of the V form, like toxicity and hæmolysin production, was greatly reduced though not completely abolished. A completely avirulent, non-toxic variant was never obtained.

*The influence of constituents of the medium on the development of the variant in culture*

Since the variant appeared readily in Koser's medium of known composition it seemed possible that by modifying the medium the variant might be suppressed and thus lead to information about the metabolic processes concerned with variation. In testing a medium, subcultures were made every two days and were plated periodically on blood-saline agar to detect variant colonies. Strains NGP 1 and 2

were used and if the V form had not appeared within 50 subcultures confirmation of this finding was sought with other strains.

*Additions to Koser's medium.* Koser's medium was tested at the same time as modifications made by adding some of the ingredients of Bordet-Gengou medium. These additions were respectively 30 per cent. of potato extract, 30 per cent. of blood and 20 per cent. each of potato extract and blood. All were solidified with 2 per cent. washed agar. Within 13 subcultures the variant had appeared on all four media.

*Subtraction of the constituent ions from Koser's medium.* The various ions present in Koser's medium were omitted one at a time in a number of media at pH 6.8-7.0, prepared by substituting one salt for another. The following stock solutions were employed, the amounts given being in grams per litre: A.R. NaCl 25.0;  $\text{NH}_4\text{H}_2\text{PO}_4$  5.0;  $\text{K}_2\text{HPO}_4$  5.0; A.R.  $\text{MgSO}_4$  1.0; A.R. sodium citrate 13.8; A.R. KCl 31.5; A.R.  $\text{MgCl}_2$  0.9;  $\text{K}_2\text{SO}_4$  0.7; A.R.  $\text{KH}_2\text{PO}_4$  7.8; A.R.  $\text{Na}_2\text{HPO}_4$  4.0;  $(\text{NH}_4)_2\text{SO}_4$  5.0; A.R. citric acid 10.0;  $\text{Na}_2\text{SO}_4$  30.4. Equal volumes of the first five gave Koser's medium when mixed together. Concentrations of the other solutions were arranged so that if an anion were omitted the concentration of the cation would be unchanged.

In the absence of ammonia or citrate there was no growth. In all the other media except those without chloride ion the variant appeared within twenty subcultures. The variant was suppressed completely on three different chloride-free media in which NaCl solution was replaced by (1) distilled water, (2) sodium citrate, (3)  $\text{Na}_2\text{SO}_4$ . The no. 1 chloride-free medium was tested thoroughly. Strains NGP 3-7, D 1, D 4 and R 1 were subcultured 150 times and strains NGP 1 and 2 over 300 times. They all remained normal in appearance of colony and by agglutination and were hæmolytic and fully toxic. All the strains tested on medium no. 1 were tested also on media 2 and 3 and remained normal for at least 50 subcultures. In subsequent experiments chloride-free medium refers simply to the omission of sodium chloride.

*pH.* No variants appeared during 100 subcultures in chloride-free medium at pH values of 5.0, 5.5, 6.8, 7.5, 9.0 and 10.0. At pH 4.5 there was no growth.

*Concentration of chloride.* A slightly modified Koser's medium was prepared in which A.R. salts were used throughout so that the approximate chloride impurity was known. A.R.  $\text{KH}_2\text{PO}_4$  was substituted for  $\text{K}_2\text{HPO}_4$  and A.R.  $(\text{NH}_4)_2\text{HPO}_4$  for  $\text{NH}_4\text{H}_2\text{PO}_4$ . The medium was constituted as follows: sodium citrate 2.77 g.,  $\text{MgSO}_4$  0.2 g.,  $\text{KH}_2\text{PO}_4$  1.0 g.,  $(\text{NH}_4)_2\text{HPO}_4$  1.0 g., NaCl varying, doubly distilled water 1 litre. The pH was 7.0 and the probable maximum chloride concentration present in the control (without added NaCl) was about  $3.0 \times 10^{-5}$  g./l.

Using strain NGP 2, the variant was not produced at a chloride ion

concentration of 0.35 g./l. or less, but appeared in the usual number of subcultures at 0.71 g./l., which was of the order of 1.0 g./l. NaCl or 0.02 *M*. Increasing the NaCl content up to double that in the usual Koser's medium had no effect on the rate of variant production.

*Effect of other anions.* Koser's medium with the 5.0 g./l. sodium chloride replaced by 5.0 g./l. of other sodium salts, all at pH 7.0, was tested, using strain NGP 2. With bromide and nitrate the variant appeared in the usual time but it did not occur with sulphate or the three sodium phosphates. With nitrite, fluoride, iodide or cyanide there was no growth even when the concentrations were lowered to 1.0 g./l., below which chloride was ineffective. The limiting value for nitrate lay between 5.0 and 2.5 g./l. and for bromide between 2.5 and 1.25 g./l., both higher than for chloride.

*Energy source.* The effect of the absence of chloride in suppressing variation was independent of the energy source in Koser's medium. A representative range of carbon compounds was substituted for the citrate at the arbitrary concentration of 4 g./l., either as free acids or sodium salts. The following groups of compounds were tested with strains NGP 2, D 1 and R 1, both in the presence and in the absence of chloride at pH 7.0: (i) glycerol, glucose, formate, acetate, pyruvate; (ii) probable decomposition products of citrate—succinate, lactate and fumarate; citrate in the same amount was used also as a control; (iii) hydroxy acids—*dl*-tartaric, *meso*-tartaric, mucic; (iv) amino acids—*dl*-alanine, *l*-cystine, asparagine; (v) dicarboxylic acids—oxalic, malonic, adipic; (vi) unsaturated dicarboxylic acids—fumaric, maleic, methyl fumaric, methyl maleic.

None of the group (i) compounds supported growth. In chloride-free media the strains were still normal after 50 subcultures with all compounds in groups ii-vi.

In the presence of chloride only three compounds suppressed the variant. These were adipic acid and the unsaturated acids maleic and methyl maleic. After 50 subcultures strains NGP 2, D 1 and R 1 were still normal. Maleic acid was tested more extensively; strains NGP 1-7 and D 4 were subcultured 100 times and NGP 2, D 1 and R 1 200 times without the appearance of the variant. This suppression of the variant by the *cis*- acid is noteworthy in contrast to the naturally occurring *trans*-isomer, fumarate, which did not have this effect. The methyl substituted *cis*- and *trans*- acids behaved similarly.

#### *Attempted reversion of the V to the N form*

No evidence of reversion was obtained during 50 subcultures of the V form of strain NGP 2 on Bordet-Gengou medium or 10 intraperitoneal passages in guinea-pigs. Similarly there was no change during 200 subcultures of the V form of strains NGP 1 and 2, D 1 and R 1 in chloride-free Koser's medium and in maleic medium with and without chloride.

*Rate of growth of N and V forms*

The quantity of a 24-hours' V culture in liquid medium adhering to a straight inoculating wire was mixed with 10 ml. of an N suspension containing about 1000 million bacteria per ml. and stronger mixtures containing one or more loopfuls of V and percentages of V from 1 to 90 were also made. Those with pure V and N suspensions were subcultured at 37° C. every two days, one loopful to 5 ml., and plated on blood-saline agar to detect the proportion of V and N colonies. The pure V and N suspensions behaved as already described.

In Koser's medium even the weakest mixture became pure V within three or four subcultures. In chloride-free medium mixtures containing up to one loopful of V did not reveal V colonies after 20 subcultures but mixtures containing two loopfuls or more of V became pure V within 3 or 4 subcultures. In maleicate medium, with and without chloride, the weakest mixture tested contained one loopful of V and this rapidly became pure V. Thus in all these circumstances the V form when initially present even in small amounts rapidly outgrew the N.

At 22° C. the V form did not rapidly outgrow the N, either in Koser's or in chloride-free medium. Starting from a mixture containing one loopful of V there were only a few V colonies after 50 subcultures and when half the initial inoculum was V there were still many N colonies.

*Increased production of the variant after irradiation by X-rays \**

Wide ranges of wave-length and dosage have been employed to obtain bacterial variation by means of X-rays (Haberman and Ellsworth, 1940; Gowen, 1941, 1942), but initial experiments indicated that dosages below 10,000 r. were not effective for *Br. bronchiseptica*. In most of the experiments a tube with a peak potential of 140 kv. (h.v.l. 1.6 mm. Cu) was used; constant potential 250 kv. (h.v.l. 1.5 mm. Cu) and 500 kv. (h.v.l. 6.3 mm. Cu) tubes gave similar results if the dosage was kept constant. The wave-lengths were respectively 0.2, 0.1 and 0.04 Å approximately and there was no wave-length effect. Dosage with back scatter was measured by means of a Victoreen dosimeter and all experiments were carried out within the homogeneous field on a phantom of pressed wood. Organisms were irradiated at a constant distance in deep Petri plates, 6 cm. in diameter, containing 5 ml. of suspension in Koser's medium or chloride-free Koser's medium, covered with sterile cellophane and sealed with wax. Suspensions were made from a 24-hours' culture on Bordet-Gengou medium inoculated with a single normal colony. They were not washed and contained  $10^8$ - $10^{10}$  viable organisms per ml. by plate counts. Determination of the effects of irradiation were begun within an hour of its completion.

After a dose of 10,000 r. the survival rate was about 1 in  $10^4$ - $10^5$  and after 20,000 r. it was from 1 in  $10^6$ - $10^7$ . It was considered that production of the variant had been stimulated if it appeared (-)

during incubation of irradiated suspension for 5 days at 37° C. or (b) during two subcultures of irradiated organisms at intervals of two days in Koser's medium, while non-irradiated controls remained normal after five subcultures.

The essential results of some experiments are given in table II for dosages of 10,000 r. and 20,000 r. Irradiation markedly hastened the appearance of the variant. Positive results were not uniformly obtained but that is not exceptional in this type of experiment.

The variant obtained after irradiation was identical with that obtained in the usual way and it outgrew the N form at 37° C.

The irradiated suspension of a single colony in chloride-free medium (strain NGP 2, expt. V, table II) was incubated at 22° C. and plated at intervals. The variant appeared within 3 days.

TABLE II

*Production of variant by X-rays*

Dosage	Medium	Occurrence of variant in the irradiated suspension incubated at 37° C. for 5 days	Occurrence of variant within two subcultures of irradiated suspension in Koser's medium
<b>Expt. I. Strain R 1</b> 140 kv., 352 r./min. { 10,000 r. 20,000 r. 10,000 r. 20,000 r. Control . . . . .	Koser minus Cl " " " Koser's medium " " " "	+ - + + -	+ + + + - at sub. 7
<b>Expt. II. Strain NGP 2</b> 140 kv., 320 r./min. { 10,000 r. 20,000 r.	Koser with and without Cl " " "	- -	- -
<b>Expt. III. Strain NGP 2</b> 250 kv., 100 r./min. { 10,000 r. 20,000 r.	Koser minus Cl " " "	- -	- -
<b>Expt. IV. Strain NGP 2</b> 140 kv., 295 r./min. { 10,000 r. 10,000 r. Controls (2)† . . . . .	Koser minus Cl (3)* Koser's medium (2)† " "	0/3 1/2 0/2	3/3 2/2 0/2 at sub. 7
<b>Expt. V. Strains NGP 2 and D 1</b> (single colonies in 10 ml. medium) 500 kv., 49 r./min. NGP 2 . . . . . 10,000 r. D 1 . . . . . 10,000 r. Controls NGP 2 (3)* . . . . . D 1 (3)* . . . . .	Koser minus Cl " " " " " " " " "	+ - 0/3 0/3	+ + 0/3 at sub. 6 0/3 at sub. 6

\* (3) = cultures exposed in triplicate.

† (2) = " " duplicate.

Irradiation of the culture medium did not increase the production of the variant when the N form was subcultured in it. Within experimental error there was no difference in the lethal effect of irradiation at 10,000 r. on normal and variant organisms.

### DISCUSSION

NaCl was shown by Winslow *et al.* (1932) to stimulate the log. phase of growth of *Bact. coli* and by Gest (1943) to produce phage-resistant strains of this organism. Large concentrations of salts have been used to produce non-hereditary variants (Matzuschita, 1900; Scales, 1921; Youmans and Delves, 1942), while lithium chloride broth has been employed to produce R variants. There have been no indications of the mode of action of salts nor has the geometrical configuration (*cis-trans*) of the energy source in relation to variation been noted.

The facts relating to the N-V change of *Br. bronchiseptica* indicate that it is the initial change which is inhibited at 37° C. by the absence of chloride and the *cis*- configuration, since the V form, once produced, outgrows N in all media. At 22° C. any V organisms would not readily outgrow the N. Hence selective influences in favour of V determine ultimately whether a pure V culture is obtained from a mixture, but the stimulation of the initial change is essential.

This initial N-V change is not related to the amount of growth in a medium; the different substrates employed with and without chloride gave rise to very different amounts of growth. The concentrations of monovalent anions necessary for the N-V change recall the influence of NaCl on the activities of some enzymes, *e.g.* amylase and mucinase, at about the same concentrations. Oppenheimer (1936) gave collected details for amylase, Br and NO<sub>3</sub> being active, although less so than Cl, while SO<sub>4</sub> and PO<sub>4</sub> were inactive. McClean and Hale (1941) and Madinaveitia and Quibell (1941) gave further examples, the latter observing a difference in the kinetics of an enzyme reaction at concentrations of 0.01 and 0.05 M NaCl. Absence or low concentration of Cl would reduce the enzymic activity involved in the initial N-V change and lead to suppression of the variant. Reduced temperature would also have this effect. There was no indication of the nature of the enzyme system involved or its relationship, if any, to the stereochemical structure of the energy source. This *cis-trans* effect suggests that the *cis*-acid interferes with the naturally occurring *trans*- enzyme system. The fact that fumarate inhibited the reduction of methylene blue by *Br. bronchiseptica* while maleicate did not, supports this view.

The mechanism of the effect of X-rays on the variation process is unknown. There is no evidence that X-rays activate enzymes and the mechanism of their effect on gene mutation, apart from accelerating normal changes, is not understood. X-rays increased the initial



production of the V form sufficiently for it to appear in the absence of chloride and at 22° C.

Additional experiments indicated that neither the concentration of chloride nor the *cis-trans* configuration had any effect on the production of variants in cultures of *Bact. aertrycke*, *Bact. friedlandcri*, *Bact. coli* or *H. parapertussis*.

### SUMMARY

Variation in *Br. bronchiseptica* (N-V), which occurred readily in culture at 37° C., appeared to depend on metabolic processes affected by NaCl concentration and the stereochemical structure of the energy source. In Koser's medium of known chemical composition variation was suppressed by NaCl concentrations below 0.02 *M* and in adequate concentrations of chloride by substituting a *cis-* for a *trans-* compound as the energy source. Bromide and nitrate had an action similar to that of chloride but the limiting concentration was higher. Results were interpreted as an effect on metabolism concerned with an initial N-V change, the production of small numbers of V organisms being followed by a rapid overgrowth of N. Irradiation with X-rays stimulated variant production apparently by affecting the initial N-V change irrespective of the culture medium.

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# A STUDY OF THE PROTECTIVE FACTORS IN HETEROSPECIFIC BLOOD GROUP PREGNANCY AND THEIR ROLE IN THE PREVENTION OF HÆMOLYTIC DISEASE OF THE NEWBORN\*

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THE phrase heterospecific pregnancy is being used with increasing frequency in current medical literature to indicate that the mother's blood contains an anti-A or an anti-B agglutinin which is incompatible with the A or B agglutinin in the red blood corpuscles of her foetus. In the opinion of the author the expression is too vague, since it might be used to denote any dissimilar feature of mother and baby. It is considered that were it to be qualified by including the factor in which mother and foetus differ, it might convey a little more to the uninitiated reader, and so the phrase "heterospecific blood group pregnancy" has been used throughout this paper.

Approximately 90 per cent. of cases of hæmolytic disease of the newborn occur in Rh-positive babies born of Rh-negative mothers (Levine *et al.*, 1941; Boorman *et al.*, 1942; Race *et al.*, 1943). Anti-Rh agglutinins stimulated in the mother pass across the placenta and cause destruction of the baby's red blood corpuscles. By contrast, although the mother's blood contains an incompatible agglutinin for an A or B agglutinin present in her foetus in twice as many pregnancies as those in which the baby is Rh-positive and the mother Rh-negative, the maternal anti-A and anti-B agglutinins are only very rarely concerned in the causation of hæmolytic disease (Boorman *et al.*, 1944). It is evident, therefore, that a protective mechanism must exist which, in the great majority of instances, prevents the anti-A and anti-B agglutinins from damaging the foetal erythrocytes, but which is more often absent in the case of the Rh factor.

An explanation of the nature of this protective mechanism has been proposed by Boorman and Dodd (1943). They consider that the maternal anti-A and anti-B agglutinins do not damage the baby's red cells because they are absorbed by water-soluble A and B group substances present in the baby's plasma and body fluids. The

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\* Part of a dissertation submitted for the degree of Doctor of Medicine in The University of Bristol.

Rh group substance, being only slightly soluble in water, is almost entirely absent from the body fluids and therefore fails to protect the foetal erythrocytes from anti-Rh agglutinins.

It has been shown, however, by Aubert *et al.* (1942) that no appreciable amount of group-specific substance is demonstrable in the plasma of approximately 50 per cent. of group A individuals and of 15 per cent. of individuals of group B. If, therefore, Boorman and Dodd's theory were correct, one would expect to find that half the number of group A babies of heterospecific blood group pregnancies are affected with hæmolytic disease. Statistically, in one pregnancy in every seven an anti-A agglutinin is present in the mother's blood incompatible with a group A or a group AB foetus, and on this basis, therefore, hæmolytic disease of the newborn should occur once in every fourteen pregnancies considering the anti-A agglutinins alone. Hence, although the corresponding group-specific substance in the baby's plasma may play a part by the inhibition of incompatible agglutinins entering the circulation, other important mechanisms must be present to protect the foetus in such heterospecific blood group pregnancies.

In an attempt to determine the nature of these protective mechanisms, the blood of a number of mothers and their babies has been examined at the time of birth.

#### TECHNIQUE

A specimen of retroplacental blood clot for the mother's blood and a sample of umbilical cord blood were collected in dry, sterile centrifuge tubes. Whenever possible a sample was also taken from the mother's arm at the time of separation of the placenta.

*Determination of blood group.* Each specimen was examined for agglutinogens in the cells and agglutinins in the serum by the tube method (see Medical Research Council War Memorandum no. 9, 1943). Negative or doubtful reactions were examined microscopically. The serum of any mother who appeared to be group B was checked against A<sub>2</sub> cells before a final diagnosis was made. In addition, if doubt existed, the saliva of baby or mother was examined for the A or B agglutininogen by the method described by Wiener (1943, p. 278).

*Titration of agglutinins in the sera.* The method outlined in M.R.C. War Memorandum no. 9 was followed. End-points were determined microscopically, for examination of sera containing anti-Rh agglutinins had shown that though agglutination was only demonstrable under the microscope it had been responsible for severe effects upon the baby. The titre recorded was the dilution of the serum before the cell suspension was added, *i.e.* the first tube of a series shows a titre of 1.

*Estimation of the group substances in the baby's serum.* One volume of the baby's cord serum was added to an equal volume of mother's serum and left to stand in the incubator at 37° C. for one hour. Parallel titrations were then set up of the mother's serum and the mixed sera, and a suspension of the baby's cells added as test cells. The difference between the end-points of the two titrations was noted after the tests had been kept at room temperature for two hours. This difference was expressed as an "inhibition index", that is, the number of times the baby's serum reduced the titre of the corresponding agglutinin in the mother's serum: *e.g.* if the mother's serum alone agglutinated

the baby's cells to a titre of 128 and the mixed sera agglutinated the same cells to a titre of 32, the inhibition index was expressed as 4 (*cf.* Aubert *et al*, 1942)

*Sensitivity of the agglutinin in the baby's erythrocytes* This has been expressed as a fraction of the sensitivity of adult cells of the same group when parallel titrations were performed with the same high titre grouping serum. Freshly made cell suspensions from a number of babies were investigated together so that the findings should be more exactly comparable

### EXPERIMENTAL FINDINGS

The blood group of 80 mothers and their babies was determined. In 20 of these pregnancies the baby was born alive to a mother whose blood contained an anti-A or anti-B agglutinin incompatible with her baby's erythrocytes

#### *The placental barrier*

The newborn infant possesses no agglutinins of its own at birth, and any agglutinins demonstrable in its serum are those of its mother which have filtered through the placenta (Smith, 1928, Polayes *et al*, 1929). Table I shows a comparison of the titre of compatible agglutinins in the mother's retroplacental blood with that in her baby's cord blood in 55 instances, and demonstrates that the placenta exerts a definite limiting effect upon the extent to which the maternal agglutinins can enter the baby's circulation. For the purpose of this paper the effect will be referred to as the "placental inhibition" of the maternal agglutinins.

Wiener and Silverman (1940) had noted this limiting effect and, from their small series of cases, concluded that it was fairly uniform in cutting down the entry of the maternal agglutinins into the foetal circulation by 8 to 16 times. This has been disputed by Sherman *et al* (1940), however, who consider that placental permeability is a very variable factor. The present investigation confirms this latter view and shows that the permeability of the human placenta to the anti-A and anti-B agglutinins varies considerably from case to case. In some pregnancies the placental inhibition is found to be as low as 8, but in another it is found that although an anti-B agglutinin is present in the mother's blood at a titre of 256, it cannot be detected in her baby's cord serum (see family no. 4). Figures for the placental inhibition as high as 64 or 128 are quite frequent findings and demonstrate that the placenta presents an efficient barrier to the entry of the maternal agglutinins into the foetal blood stream in many pregnancies.

#### *Retroplacental inhibition*

A marked difference between the titre of the compatible and incompatible agglutinin in the retroplacental blood was observed in five of the heterospecific blood group pregnancies examined, the titre of the incompatible agglutinin being greatly diminished (table I,

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families 1, 8, 32, 51 and 68). In those cases in which a specimen of the mother's intravenous blood had also been obtained there was little difference between the titre of the two agglutinins (family no. 32, tables I and II). Hirsfeld and Zborowski (1925) have also observed this difference and suggest that it is indicative of the presence of a specific neutralising mechanism in the foetus. They are of the opinion that the lowering of the titre of the potentially dangerous agglutinin in the retroplacental region, together with the lack of permeability of the placenta to agglutinins, is the physiological mechanism that enables the foetus to survive a heterospecific blood group pregnancy. The concentration of the incompatible agglutinin in the mother's general circulation, as indicated by blood taken from the ante-cubital fossa, may, however, be 32-64 times greater than that in a retroplacental specimen taken at the same time. The difference is so great that if Hirsfeld and Zborowski's explanation were correct, i.e. that this process is concerned in the natural protection of the foetus throughout pregnancy, it would surely find reflection in the mother's general circulation. As an alternative, it may be suggested that this phenomenon is not a part of the protective mechanism, but is the result of accidental contamination of the retroplacental blood by the baby's group substances at the time of separation of the placenta. Occurring at this time, when the retroplacental blood has ceased to have any communication with the general circulation, this marked fall in titre would not be found in blood taken from the mother's ante-cubital fossa.

#### *The group substances in the baby's plasma*

In 7 of the 20 heterospecific blood group pregnancies no group substances could be demonstrated in the baby's serum (table II), but in none of these cases did the baby develop frank hæmolytic disease of the newborn.

When investigating the protective action of the plasma agglutinin following the transfusion of incompatible agglutinins, Boorman and Dodd found that the absorption of antibody by even small amounts of plasma agglutinin was considerable. While the present findings do not belittle the assumption that the group-specific substances may be potent factors in the absorption of incompatible agglutinins crossing the placental barrier, they do demonstrate clearly that plasma agglutinogens do not play a major part in the prevention of hæmolytic disease of the newborn.

#### *The sensitivity of the foetal erythrocytes to agglutination*

It has been shown by Kemp (1930) that the erythrocytes of the newborn have only 20 per cent. of the sensitivity to agglutination of adult cells. This observation suggests that a low sensitivity of the baby's cells is a part of the natural protective mechanism.

TABLE I

*The placental inhibition of the maternal anti-A and anti-B agglutinins*

Family no.	Mother's group	Baby's group	Titre of agglutinins				Placental inhibition	
			Mother's blood (retroplacental)		Baby's blood		Anti-A	Anti-B
			Anti-A	Anti-B	Anti-A	Anti-B		
1	O	A	8-16	128	...	2	...	64
2	A	O	...	512	...	16	...	32
3	A	A	...	64	...	0	...	>64
4	A	A	...	256	...	0	...	>256
5	O	O	1024	1024	32	16	32	64
6	O	O	512-1024	128	16	4	32-64	32
7	O	O	512	128	4	2	128	64
8	O	A	16	128-256	...	2-4	...	64
9	B	B	32-64	...	2	...	16-32	...
10	A	O	...	64	...	0	...	>64
11	O	A	256	512	...	8	...	64
12	O	A	256	256	...	4	...	64
13	O	A	256	64	...	0	...	>64
14	O	A	1024	128	...	0	...	>128
15	O	O	256	128	8	8	32	16
16	A	A	...	32	...	2	...	16
17	O	O	1024	256	16	4	64	64
19	O	O	512	128	64	16	8	8
20	O	O	1024	256	64	1	16	256
21	A	O	...	128	...	1	...	128
22	O	B	64	16	8	...	8	...
23	O	A	32	64	...	0	...	>64
24	A	A	...	256	...	2	...	128
26	A	A	...	64/128	...	0	...	>64/128
27	A	A	...	32	...	0	...	>32
28	O	O	128	256	8	16	16	16
29	O	O	256	256	8	8	32	32
32	O	A	4	512	...	32	...	16
33	O	O	128	128	0	0	>128	>128
34	A	O	...	256	...	0	...	>256
35	A	A	...	256	...	2	...	128
36	O	O	256	256	4	8	64	32
37	B	B	128	...	16	...	8	...
38	A	A	...	512	...	2-4	...	128-256
39	A	O	...	64-128	...	0	...	>64-128
40	A	O	...	64	...	0	...	>64
41	A	A	...	128	...	4	...	32
42	O	O	128	64	4	0	32	>64
46	A	A	...	256	...	4	...	64
47	O	A	16	32	...	4	...	8
50	O	A	256	128	...	2	...	64
51	O	A	8	64	...	4	...	16
53	O	O	128	64	1	0	128	>64
56	O	O	512	128	8	2	64	64
63	A	A	...	64	...	0	...	>64
64	O	A	512	512	...	8	...	64
66	O	O	128	64	0	0	>128	>64
67	A	O	...	64	...	1	...	64
68	O	B	512	64	8	...	64	...
69	A	A	...	256	...	8	...	>64
70	A	O	...	64	...	0	...	8
71	A	A	...	128	...	16	...	>64
72	A	A	...	64	...	0	...	...
77	B	O	128	...	8	...	16	...
80	O	O	256	256	1	1	256	256

> = at least.

A detailed study of individual cases reveals that Kemp's generalisation is very misleading. In fact, the sensitivity to agglutination of the erythrocytes varies greatly from baby to baby. In one it may be only  $\frac{1}{3}$  of that of the adult, while in another it is equal to that of strongly reacting adult cells (table II). Hence it is evident that, although a lack of sensitivity of the baby's erythrocytes may be a protective factor in some pregnancies, it is by no means invariably so.

#### *The effect of temperature*

The supposition that the mother's blood contains anti-A or anti-B agglutinins capable of damaging her infant's erythrocytes has been based upon titrations usually performed at room temperature. In general their titre at body temperature ( $37^{\circ}\text{C}.$ ) is but  $\frac{1}{2}$  to  $\frac{1}{3}$  that at room temperature (Wiener, p. 18), so that if this fact is ignored the in-vivo activity of the agglutinin may tend to be over-estimated. Again, in the present investigation the effect of temperature has differed from case to case, but in general the mother's serum did not agglutinate the baby's red cells as strongly at body temperature as at room temperature (table II).

#### DISCUSSION

It is now possible to assess the role of the main factors in providing protection to the foetus in the 20 cases of heterospecific blood group pregnancy which were examined.

Previous investigations concerned with the part played by the Rh factor in the pathogenesis of hæmolytic disease of the newborn have been restricted to classical cases of the disease (Levine *et al.*; Boorman *et al.*, 1942; Race *et al.*). For the present investigation it was decided that only such cases should be considered as indicative of a failure of the normal protective mechanism in heterospecific blood group pregnancy. All the babies in our series were born alive, none gave occasion for anxiety from jaundice, œdema or anæmia for at least 14 days after birth, and all were considered to be making normal progress at a post-natal examination one month after leaving hospital. In addition, a midwife reported that the baby's progress was satisfactory during the first 5-6 months of its life. It may therefore be presumed that all these babies received adequate protection against their mother's anti-A and anti-B agglutinins whilst *in utero*.

Because it is not uniform, the placental inhibition of the incompatible agglutinin cannot be pre-determined for any particular case. It is found, however, that where compatible anti-A and anti-B agglutinins are together present in the mother's blood (*i.e.* group O mother, group O baby) the permeability of the placenta to both agglutinins is usually approximately equal. Hence the extent to which the incompatible agglutinin has crossed the placental barrier



may be estimated with some certainty in those pregnancies where the mother is group O. This was possible in 14 of the 20 pregnancies. Moreover, since the titre of the incompatible agglutinin differed little from that of the compatible agglutinin, both in the specimens of intravenous blood examined and in many of the samples of retro-placental blood, it may be concluded that the titre of the compatible agglutinin in the baby's blood is an index of the entry of the dangerous agglutinin into the baby's circulation. In only one pregnancy (family 32) was it found that a compatible agglutinin was present in the baby's blood in any marked titre (32). The titre of this antibody in the other babies varied from 0 to 8, despite the fact that the corresponding titre in the maternal blood was high (see table II). This clearly demonstrates the importance of the placental barrier in the protection of the foetus in heterospecific blood group pregnancy.

The protection afforded by the relative sensitivity of the baby's erythrocytes to agglutination may also be assessed with some certainty and the results suggest that the combined effect of the relative impermeability of the placenta and lack of sensitivity of the baby's corpuscles to the maternal agglutinins is sufficient to protect the baby's erythrocytes in many such pregnancies.

The full extent of the part played by the group substances in the baby's plasma can only be surmised, for sufficient evidence is not yet available to permit of its exact evaluation. It may be presumed, however, that in those cases in which the combination of the above two factors does not appear to have been sufficient, the agglutinins were prevented from producing disastrous effects by the presence of these known neutralising agents.

Similarly, too, in the present state of our knowledge, no definite conclusion can be reached on the extent to which body temperature limits the activity of incompatible anti-A and anti-B agglutinins *in vivo*. The results of the present investigation do suggest, however, that although the total protection afforded by the first three factors probably gives a wide margin of safety in most heterospecific blood group pregnancies, this physiological mechanism may be still further enhanced by the depression of the activity of the agglutinins *in vitro*.

### *Protection against anti-Rh agglutinins*

Nearly every case of hæmolytic disease of the newborn is due to anti-Rh agglutinins, hence it should be possible to demonstrate an absence or failure of protective factors against these antibodies.

When the father is homozygous for the Rh factor, the demonstration of anti-Rh agglutinins in the mother's blood during the ante-natal period provides almost certain evidence that her foetus will be affected with hæmolytic disease (Boorman *et al.*, 1944), and yet many cases are on record in which these antibodies have been found in only low titre in the mother's serum. A rapidly fatal case, with the

diagnosis confirmed by necropsy, has been recorded in which anti-Rh agglutinins were not detectable in a titre greater than 1-2 in the mother's blood (Langley and Stratton, 1944), and Boorman *et al.* (1942) report that in 19 cases of established hæmolytic disease of the newborn the maternal agglutinins did not rise above a titre of 8. It must also be remembered that these findings have been observed during the post-partum period when immune agglutinins attain their maximal strength (Boorman *et al.*, 1942). In none of the 55 pregnancies investigated in the present series would the anti-A or anti-B agglutinins have passed across the placenta into the baby's circulation had they been present in the mother at a titre of less than 8. More frequently the titre had to attain to 64 or 128 before these agglutinins appeared in the umbilical cord blood. This suggests that in most pregnancies there is little or no barrier to anti-Rh agglutinins and that these antibodies pass across the placenta more freely than the anti-A and anti-B agglutinins.

Having gained access to the foetal circulation, the Rh antibody is not prevented from damaging the erythrocytes by the three other factors that protect the foetus in a heterospecific blood group pregnancy, for no Rh group-substance is present in the baby's plasma (Boorman and Dodd), the Rh agglutinin is usually well developed at the time of birth (Wiener, p. 252), and the maternal anti-Rh agglutinins in general react most intensely at body temperature (Levine *et al.*; Boorman *et al.*, 1942).

The conclusion may be drawn that an absence, either partial or complete, of any mechanisms comparable to those that protect the foetal erythrocytes from destruction by the anti-A and anti-B agglutinins probably explains why Rh antibodies stimulated in the mother have such damaging effect upon her baby.

Several investigators (Gallagher *et al.*, 1943; Wiener and Wexler, 1943; Langley and Stratton; Mollison, 1944) have noted the absence of any close relationship between the titre of the anti-Rh agglutinins in the mother's blood and the extent and rapidity of destruction of the red cells of the baby in hæmolytic disease. If the wide range of variation in the placental inhibition of anti-A and anti-B agglutinins is any criterion, this is not surprising. Moreover, the effects in the baby will further depend upon the degree of absence of the other protective mechanisms.

#### SUMMARY AND CONCLUSIONS

No one factor is wholly concerned in the protection of the foetal erythrocytes from the action of the maternal antibodies in a heterospecific blood group pregnancy, but probably different combinations of at least four main mechanisms operate, namely:—

(i) lack of permeability of the human placenta to the anti-A and the anti-B agglutinins;

(ii) neutralisation of incompatible anti-A or anti-B agglutinins crossing the placental barrier by group-specific substances in the foetal plasma ;

(iii) lack of sensitivity of the foetal erythrocytes towards the incompatible agglutinins ; and

(iv) diminished activity of the anti-A and the anti-B agglutinins at body temperature.

It is suggested that the important part played by the Rh agglutininogen and its corresponding agglutinin in the pathogenesis of hæmolytic disease of the newborn is in part due to the freer passage of the anti-Rh agglutinins across the placenta, relative to the anti-A and the anti-B agglutinins.

It would appear that, in general, little can be done to enhance the natural protective mechanism in women known to be Rh negative. It is within our power, however, to avoid increasing the titre of the anti-Rh agglutinins in the mother's blood by ensuring that the antigenic Rh factor is not needlessly introduced into the circulation of any Rh-negative female who is either an actual or a potential child-bearer. Dacie and Mollison (1943) and Cappell (1944) have shown that it is possible to become sensitised to the Rh antigen by a single transfusion. Therefore from the cradle every female should be Rh typed, if circumstances permit, before receiving a blood transfusion.

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## THE NON-MANNITOL-FERMENTING DYSENTERY BACILLI

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SINCE Shiga's original discovery of a "Ruhr-bazillus" in 1898 (Shiga, 1898, *a*, *b*) only one other bacterium with comparable cultural characters and fermentative activities has received general acceptance as a cause of acute epidemic dysentery in man. This is the organism first isolated by Schmitz in 1917 during the investigation of an outbreak among prisoners of war in Rumania. The two species—*Bact. dysenteriae* Shiga and *Bact. dysenteriae* Schmitz—differ from the other dysentery bacilli in their inability to ferment mannitol: each, however, is antigenically distinct and, so far as their agglutinogens are concerned, antigenically homogeneous. Yet from time to time reports have appeared of non-mannitol-fermenting bacilli of the general *Shiga-Schmitz* type, which, even on primary isolation from cases of clinical dysentery, have failed to react with anti-*Shiga* or anti-*Schmitz* agglutinating sera. The possibility that some of these inagglutinable strains may have been rough variants cannot be entirely excluded, but in recent years such an explanation has become far less tenable. It is the purpose of this paper to describe some of these rarer dysentery organisms and to assess their position and significance in the light of modern work on the group as a whole.

### *Material and methods*

In the Middle East Force from 1940 to 1942 it was the routine laboratory practice to attempt to isolate and identify the pathogen in all cases of dysentery and diarrhoea admitted to hospital. Through the courtesy of the Deputy Director of Pathology, M.E.F. (Colonel J. S. K. Boyd, O.B.E.), arrangements were made for all inagglutinable strains which failed to ferment mannitol to be subcultured on Dorset egg medium and forwarded direct to the writer for further study. Between August 1940 and September 1942 (when the investigation had to be terminated owing to pressure of other duties) 304 live strains were received. Usually adequate clinical details accompanied the cultures, but in only a fraction of the cases was it possible to examine the patients themselves. Early in 1941 representative cultures of inagglutinable non-mannitol-fermenting bacilli were received from India from Lieut.-Colonel A. Sachs, R.A.M.C., who had been investigating the group there. A small number of strains have also been obtained from sources outside the Middle East. Finally, during 1941 and part of 1942, a collection of 221 cultures of *Bact. dysenteriae* Shiga and 98 of *Bact. dysenteriae* Schmitz was accumulated for comparison.

On receipt, each culture was given a code number, re-plated for purity and smoothness and then inoculated into nutrient broth, nutrient gelatin, litmus milk, peptone water, and the following carbohydrate media: glucose, lactose, sucrose, rhamnose, arabinose, xylose, mannitol and dulcitol. These were incubated aerobically at 37° C. for three weeks, and throughout this period the motility of the strain, its ability to produce indole, its proteolytic powers and its fermentation reactions were examined routinely and repeatedly. If on the results it was decided that the organism did in fact belong to the dysentery group, preliminary agglutination tests were carried out with antisera prepared against strains of *Bact. dysenteriae* Shiga, Schmitz, Sonne, Flexner I-VI, Boyd I-III and *Bact. alkalescens*.

Subsequently representative strains were selected for the purpose of producing anti-sera in rabbits. As a rule little difficulty was encountered in obtaining anti-sera of titre 1:640 or higher and no sera of lower titre were employed. The anti-sera were stored in the refrigerator without preservative.

## RESULTS

No less than 67 of the cultures were discarded as belonging to the *Proteus morgani* group. Usually it was easy to identify these organisms by their motility, their colonial characters and their production of gas in sugar media. In all cases of doubt subcultures were made on sloppy (1 per cent.) agar and examined for swarming (Rauss, 1936; Sevin and Buttiaux, 1939). It is perhaps worth pointing out that some strains of *P. morgani* fail to produce gas from fermentable carbohydrate media, even after prolonged incubation, and that strains producing only a minute bubble in the first 24-48 hours are by no means uncommon. On the other hand, true non-motile variants must be excessively rare, at any rate on fresh isolation. I have never encountered any, and Sevin and Buttiaux mention only one strain in their very complete record. But motility is not always easy to demonstrate; prolonged and repeated examination may be necessary before even a single motile cell is found.

Quite apart from these interlopers, 20 strains proved to belong to recognised types of dysentery bacilli. Five Shiga and two Schmitz strains require no further mention; the remaining 13 were non-mannitol-fermenting variants of the Flexner-Boyd group and belonged to the following serological types: (88)—3 strains; (103)—6 strains; (D 1)—4 strains.

Such variants, though by no means common, have been described before (Clayton and Warren, 1928-29, 1929-30; Boyd, 1930 '40; Sachs, 1943) and were not further investigated.

There remained 217 strains which could not be were Gram-negative, non-motile bacilli, fermenting without gas, but not lactose or mannitol; and on 1 per cent. agar they gave discrete type of growth. The cultural reactions several times and gave consistent result that the vast majority of these Middle types already identified and named in Inc

than confuse an already overburdened literature, Sachs's designations, when applicable, have been adopted throughout.

*Cultural characters of the non-mannitol-fermenting strains*

The colonies of these organisms on nutrient agar, on McConkey and on litmus-lactose-bile salt-agar are small, round, discrete and rather flat; they are transparent and often have a delicate bluish sheen, but with age certain strains become more opaque. This is especially evident with type A 12 (Sachs) and the one strain of type B 105 (Sachs) which I have studied, but is also to be seen to a lesser degree with types B 81 (Sachs) and P 25 (MacLennan). In no instance, however, are the colonies likely to be mistaken for those of *P. morgani* or of the majority of paracolon and *alkalescens* strains, which are altogether larger and coarser.

The biochemical reactions of the strains are shown in table I, in which only those of significance are included. From this it will

TABLE I

*Non mannitol fermenting dysentery bacilli: biochemical reactions*

Strain	Glucose	Lactose	Sucrose	Mannitol	Dulcitol	Rhamnose	Indole
Shiga	A 1	Nil	Nil	Nil	Nil	Nil	—
Q 1167	A 1	Nil	Nil	Nil	Nil	Nil	—
Q 771	A 1	Nil	Nil	Nil	Nil	Nil	—
Q 454	A 1	Nil	Nil	Nil	Nil	Nil	—
A 12	A 1	Nil	Nil	Nil	Nil	Nil	—
Q 1030	A 1	Nil	Nil	Nil	A 1-3	Nil	—
Schmitz	A 1	Nil	Nil	Nil	Nil	A 1	+
Q 902	A 1	Nil	Nil	Nil	Nil	A 1	+
P 25	A 1	Nil	A 2	Nil	Nil	Nil	+
B 81	A 1	Nil	A 3-6	Nil	Nil	Nil	+
B 105	A 1	Nil	A 3	Nil	Nil	Nil	+

A 1-3 = acid produced in 1-3 days.

be seen that these organisms may be readily classified into three groups according to their action on dulcitol and their ability to produce indole. The dulcitol-negative, indole-negative group includes *Bact. dysenteriae* Shiga, and types Q 1167, Q 771, Q 454 and A 12 of Sachs; the dulcitol-negative, indole-positive group, *Bact. dysenteriae* Schmitz, types Q 902, B 81 and B 105 of Sachs and P 25 of the writer. Type Q 1030 (Sachs) occupies a class by itself, being indole-negative and a consistent late fermenter of dulcitol. A further subdivision of the *Bact. dysenteriae* Schmitz sub-group into those that attack sucrose and those that do not scarcely seems called for.

I have been unable to confirm completely Sachs's findings concerning the fermentation of arabinose. He regards this sugar as of great importance in the classification of the group. Although the reactions reported by Sachs are broadly true, they are not quite so



definite as he indicated. Many strains of Q 1167 will attack arabinose and several of the so-called "arabinose-positive" types—particularly Q 454 and A 12—are without action, while in others the fermentation has been so delayed and irregular as to render its use in diagnosis of very doubtful value. I should, however, like to emphasise the usefulness of rhamnose in this respect. The only two organisms to attack rhamnose have been *Bact. dysenteriae* Schmitz and type Q 902. This reaction has been tested out in the entire collection of 221 strains of *Bact. dysenteriae* Shiga, 98 of *Bact. dysenteriae* Schmitz and 217 of other types, and has been consistent.

### Serological investigations

Anti-sera were prepared in rabbits against each representative type and every strain was tested routinely against all the anti-sera. Results, read after 4 hours' incubation at 56° C. and again after standing on the bench overnight, were clear-cut and simple to read. It can be said at once that, with one exception, the various types were serologically homogeneous and distinct. It is true that there was occasionally some cross agglutination to a titre of 1:10 to 1:40 between *Bact. dysenteriae* Shiga and *Bact. dysenteriae* Schmitz, between *Bact. dysenteriae* Shiga and Q 1167, and between *Bact. dysenteriae* Schmitz and Q 902, but this was so slight and irregular that it was not considered significant. The exception was found among some strains of type Q 771, which showed some antigenic complexity.

The original anti-serum to Q 771 had been prepared not against one of Sachs's strains but against a Middle East strain—"McDiarmid". Subsequent cross absorption tests, however, showed that McDiarmid, although very similar to Sachs's Q 771, was not identical (table II).

TABLE II

*Cross absorption experiments with anti-sera Q 771 and McDiarmid*

Anti-serum	Absorbing suspension	Agglutinating titre with suspension	
		Q 771	McDiarmid
Q 771	Nil	1:640	1:640
"	Q 771	Nil	Nil
"	McDiarmid	Nil	Nil
McDiarmid	Nil	1:1280	1:1280
"	Q 771	Nil	1:320
"	McDiarmid	Nil	Nil

These findings were thought so unusual that fresh anti-sera were prepared in two other rabbits, with exactly similar results. Thereafter each strain of Q 771-McDiarmid type was tested out against both anti-sera and absorption tests were undertaken as a routine. In all 103 such strains were examined, of which 95 were of mixed

Q 771-McDiarmid type, and 8 (strains PL 4, PM 2, PM 7, PR 1, P 19, P 37, P 67 and P 89) were of pure McDiarmid type. Attempts to isolate heterologous variants from either sub-type were not successful but were not thoroughly pursued.

None of the other organisms studied, apart from those already mentioned, showed any antigenic relationship with the Boyd-Flexner or Sonne types of dysentery bacilli.

### *Occurrence of the various types*

These organisms have been obtained from sources scattered throughout the Middle East command: no significant geographical incidence can be discerned in their distribution.

*Frequency of non-mannitol-fermenting strains.* There is, unfortunately, no completely accurate information on the absolute frequency of these organisms in the M.E.F. It is true that throughout the period under review figures are available of the total isolations of dysentery bacilli in the M.E.F. laboratories (table III), but the

TABLE III

*Isolation of dysentery bacilli in the M.E.F., August 1940-September 1942*

Organism	Total Isolations	Percentage
<i>Bact. dysenteriae</i>		
Shiga . . . . .	3,181	17.9
Schmitz . . . . .	1,223	6.8
Flexner . . . . .	10,929	61.5
Sonne . . . . .	1,284	7.2
<i>Inagglutinable strains</i>		
Mannitol-fermenters . .	741	4.2
Non-mannitol-fermenters .	443	2.4

figure given for inagglutinable non-mannitol-fermenters (443) cannot be accepted unequivocally. It will be recalled that only 304 of these 443 strains were actually received and found suitable for detailed study, and that not all of these were in fact dysentery bacilli. If our series of cultures was representative, then 310 of the 443 so-called inagglutinable non-mannitol-fermenters would have been genuine, giving an over-all incidence of 1.7 per cent., but this is mere conjecture.

*Relative frequency of types in the non-mannitol-fermenting group.* This is set out in table IV.

### *Pathogenicity*

It was pointed out by Shiga (1898b), and again more explicitly by Boyd (1940), that to satisfy the so-called postulates of Koch is a matter of peculiar difficulty in the case of the dysentery bacilli, and these difficulties have been increased rather than lessened by the introduction in recent years of more refined and specific culture

media. It is just because of this that many of the older accounts of cases and even of epidemics of clinical dysentery due to "in-agglutinable Shiga bacilli" and the like cannot be accepted unreservedly. On the other hand the evidence of pathogenicity presented by Dudgeon and Urquhart (1919) and Mohamed Ali (1938) for the "Para-Shiga (—)" strains, by Silva (1943) for inagglutinable organisms of the Shiga-Schmitz type, by Christensen and Gowen

TABLE IV

*Relative frequency of serological types among 217 strains of non-mannitol-fermenting bacilli*

Type	No. of strains	Type	No. of strains
Q 1167	37	Q 454	16
Q 1039	23	Q 902	4
Q 771/McDiarmid	103	A 12	8
Mixed 95		B 81	7
McDiarmid 8		P 25	19

(1944) for some late arabinose fermenters, by Gober, Stacy and Woodrow (1944) for "a new type of non-mannitol fermenting Shigella", by W. H. Ewing (personal communication) for certain strains isolated from the U.S. forces in the Mediterranean area, and particularly by Archer (1933), Large and Sankaran (1934), Boyd (1935) and Sachs (1943) for Indian strains of non-mannitol-fermenting organisms, leaves little doubt of the ætiological importance of such strains in dysenteric infections.

In the Middle East the evidence that can be brought forward is at best circumstantial and confirmatory, for it must be remembered these cases were occurring in an active theatre of military operations. In consequence it was rarely possible to follow a case throughout his infection, or indeed even to examine him personally. The following points do, however, seem worthy of emphasis.

1. Although throughout the period under review numerous so-called normal stools were being examined routinely in the control of cooks and foodhandlers, these organisms have been isolated only from pathological stools from cases of dysentery or diarrhoea, and in some instances the infection has been severe. Sufficiently full details have been obtained from 119 of the 217 Middle East cases to give some idea of the type of disease with which the various organisms have been associated (table V).

2. Apart from B 105, all of Sachs's types have been identified in the Middle East, although their relative incidence has differed from that reported in India. Moreover, it is known that certain of Sachs's types have been isolated from cases of clinical dysentery in West Africa (N. Martin, personal communication), and a strain recovered from a case of dysentery in Rabaul in New Guinea and sent

to me by Major Backhouse, A.A.M.C., proved to belong to type Q 1167. The one new type—P 25—not defined by Sachs, has since been identified as the presumptive cause of an epidemic of institutional dysentery among infants in England. (I have to thank Dr D. M. Stone for sending me these strains.) Two small outbreaks of dysentery were also studied in the Middle East in which the only abnormal organisms were Q 1030 and P 25 respectively, and these organisms were found consistently in the affected cases.

TABLE V

*Nature of infection produced by 119 strains of non-mannitol-fermenters*

Organism	No. of cases	Type of exudate			Severity of infection		
		Bacillary	Indefinite	Nil	Mild	Moderate	Severe
Q 1167	20	17	3	0	2	12	6
Q 1030	11	8	3	0	7	4	0
Q 902	1	0	1	0	1	0	0
Q 771	67	51	14	2	56	10	1
Q 454	7	3	4	0	7	0	0
A 12	3	3	0	0	3	0	0
B 81	2	1	1	0	2	0	0
P 25	8	7	1	0	5	3	0
Total . .	119	90	27	2	83	29	7

Two of these cases showed multiple infections, one with *E. histolytica* and Q 1030, the other with *Bact. dysenteriae* Flexner and Q 771: both had a bacillary type exudate and both were described as having moderately severe infections.

It is felt that these observations, although admittedly inconclusive in themselves, materially strengthen the case for regarding these organisms as true pathogens, capable of producing dysentery in man.

### DISCUSSION

The first attempt to classify the dysentery bacilli (Hiss, 1904-05) made use of their action on mannitol as a primary criterion and this has since been generally adopted. It was soon realised, however, that the mannitol-fermenting strains represented a complex mixture, which could not be sorted out merely by recourse to further fermentation reactions (Kruse *et al.*, 1907). More precise classification seemed to depend upon an antigenic analysis of the organisms (Murray, 1918; Gettings, 1919; Andrewes and Inman, 1919), and this was indeed the method finally employed by Boyd (1940) in his definitive studies. As for the non-mannitol-fermenters, it was long believed that *Bact. dysenteriae* Shiga was the sole representative, and even with the recognition of *Bact. dysenteriae* Schmitz as a separate species (Schmitz, 1917; Andrewes, 1918) a new classification on serological grounds, though perfectly feasible, was scarcely necessary, for the two organisms could be readily differentiated by testing for their ability to produce indole and by their action on rhamnose.

Now, however, the position has entirely changed. At least nine new types of dysentery bacilli, concerning whose relationship to the disease there can be little doubt, have been identified and defined, and none can be differentiated by biochemical reactions from *Bact. dysenteriae* Shiga or *Bact. dysenteriae* Schmitz. Unfortunately, apart from the Indian and Middle East strains (and to a lesser degree the West African) no correlation between the various new species has so far been attempted; but before any new classification is embarked upon, some cross identification seems to be essential. It is true that in the Middle East these organisms comprised at most only 2 per cent. of all the dysentery bacilli isolated and 9 per cent. of the non-mannitol-fermenters, but they appear to be sufficiently widely spread to justify their inclusion in some new scheme. Obviously they cannot be classified simply as varieties of *Bact. dysenteriae* Shiga and *Bact. dysenteriae* Schmitz, but, with the exception of Sachs's Q 1030, they might well be grouped together as specific types of *Bact. dysenteriae* parashiga and *Bact. dysenteriae* paraschmitz. Q 1030, on the other hand, does not fit readily into such a scheme, and will probably have to stand on its own.

### SUMMARY

1. Certain strains of non-mannitol-fermenting bacilli isolated from cases of dysentery and diarrhoea in the Middle East have been described.

2. In addition to 221 cultures of *Bact. dysenteriae* Shiga and 95 of *Bact. dysenteriae* Schmitz, 304 unidentified cultures were studied, of which 217 appeared to be true dysentery bacilli.

3. These dysentery organisms could be classified into 3 groups by their cultural reactions in glucose, dulcitol and peptone water.

4. Of the 8 serological types described by Sachs, 7 were identified in the present series; 19 strains not belonging to Sachs's types were shown to belong to a new serological type.

5. Some evidence of the pathogenicity of these organisms has been produced.

This study could not have been undertaken without the co-operation of a large number of pathologists, in the Middle East and elsewhere, to all of whom I offer my sincere thanks. I should also like to place on record my indebtedness to Colonel J. S. K. Boyd for much advice and encouragement, and to Mrs E. M. Robinson for technical assistance.

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A HISTOLOGICAL STUDY OF THE LUNGS OF MICE  
INFECTED WITH TYPHUS RICKETTSIÆ

W. NYKA

*From a Military Laboratory*

(PLATES XXXVIII-XLI)

SINCE Castaneda (1938, 1939) first succeeded in infecting mice with typhus by the intranasal introduction of suspensions of rickettsiæ into the lungs, this method has been used as a routine for growing the typhus virus. In subsequent years mice have been inoculated in large numbers, yet little has been done to establish the nature of the inflammatory process caused by the rickettsiæ or to see what appearance these organisms have within the lesions they provoke. Indeed, in the literature of typhus, so far as it is obtainable at the present time, no histological study of the experimental pneumonia in mice infected with typhus could be found. It therefore seemed worth while to undertake such a study and to try to determine the anatomical basis of this type of experimental typhus in animals.

*Methods*

The suspension used for the infection of the animals was prepared as follows. The lungs of mice killed with chloroform on the third or fourth day after inoculation were ground up with 10 per cent. of serum-broth in the proportion of 2.4 c.c. per lung. The suspension thus obtained was spun down for five minutes at 1000 r.p.m. The supernatant fluid was removed and used either fresh or after storage at  $-76^{\circ}$  C. in a mixture of alcohol and solid carbon dioxide. The dose used varied in quantity as well as in concentration. Certain batches of animals received 0.05 c.c., some 0.075 c.c. and others 0.1 c.c. of full strength suspension. Sometimes the concentrated suspension was diluted with an equal volume of serum-broth. Three murine and seven epidemic strains were used in this study.

The mice recover from the combined effects of anæsthetic and inoculation within 3 or 4 hours and show no symptoms until a few hours before death, when the respiratory rate increases, a bronchial type of respiration develops, the fur becomes ruffled and the response to external stimuli greatly diminished.

Finally, the various stages of evolution of the lesions, leading to almost complete hepatisation.

Microscopical sections for the demonstration of rickettsiæ in the lungs were prepared either by the method previously described (Nyka, 1944) or an improved method, still however experimental, carried out at present as follows.



1. Fix in 10 per cent. neutral formol.
2. Stain for 30 minutes to 1 hour in 1 : 10,000 aqueous methyl violet.
3. Differentiate in weak acetic acid (2 drops of glacial acetic acid in 100 ml. water), controlling the differentiation microscopically until cell cytoplasm is unstained.
4. Counterstain with 1 : 10,000 aqueous metanil yellow for a few seconds.
5. Dehydrate with acetone, clear in xylol and mount in DPX 4 mounting medium (Media Manufacturing Centre) or in Xam (Gurr).

For showing the histological changes in the lungs sections were stained with hæmatoxylin and eosin.

### MORBID ANATOMY

To obtain a good view of the pleural cavity, the thorax of the mouse is opened from the dorsal aspect. In mice which die spontaneously the whole lung is dark red, considerably enlarged and entirely consolidated, with a moist smooth surface. There are no pleural adhesions but very often the pleural cavity contains a small quantity of yellow sero-fibrinous fluid. In mice killed with chloroform the picture is somewhat different, and after examining several batches of fifty animals, lesions in various stages could be made out.

In animals with severe lesions the consolidation involves the whole lung, only part of the edges of the lower lobes being still aerated. In those with slighter lesions consolidation is limited to the upper part of the lung, while the base is congested only, or congested and studded with a variable number of foci of consolidation. Usually both lungs present the same degree of involvement, but about 5 per cent. of the large number of mice examined show unilateral consolidation, the other lung being congested and the site of a few large or of a greater number of small foci of consolidation. Usually the left lung is the more severely affected. The degree of consolidation varies and the demarcation between consolidated and non-consolidated lung may be either sharply or ill defined. Lack of definition is due to the presence of a transitional area of partial consolidation. In the lumen of the trachea a small quantity of sero-fibrinous fluid is found and the mucosa of both trachea and large bronchi is markedly hyperæmic, deep red and velvety.

### MORBID HISTOLOGY

Within the congested but non-consolidated lung (fig. 1) the most striking change is dilatation of all blood vessels, but mainly of the capillaries of the alveolar walls. The epithelial cells lining the alveoli are somewhat swollen, causing narrowing of the lumen. Such congestion with only slight swelling of the alveolar cells may involve whole lobes, which show no consolidation. Usually in addition to simple congestion, patches of consolidation are found consisting essentially of thickened inter-alveolar septa and a variable number of leucocytes.

## EXPERIMENTAL TYPHUS PNEUMONIA IN MICE



FIG. 1.—Epidemic typhus. Marked congestion. Haematoxylin and eosin.  $\times 70$ .



FIG. 2.—Murine typhus. Congestion, with exudate. Haematoxylin and eosin  $\times 70$ .

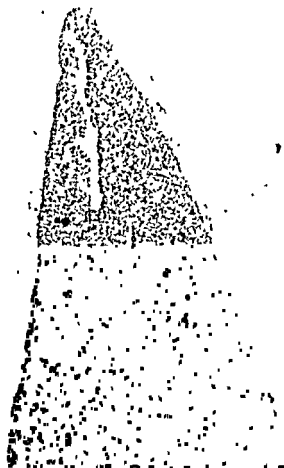


FIG. 3.—Murine typhus. Complete consolidation, caused mainly by swelling of the alveolar cells. Haematoxylin and eosin  $\times 70$ .



FIG. 4.—Murine typhus. Complete consolidation, showing swelling of the alveolar cells and exudate filling the alveoli. Haematoxylin and eosin  $\times 70$ .



Consolidation of the lung may be brought about either by an inflammatory œdema (fig. 2) or by swelling of the alveolar epithelium, while desquamated alveolar cells and occasionally a variable number of leucocytes are present in the lumen (fig. 3). Although lesions of the latter type may involve a lobe or even a whole lung, they usually coexist with lesions of the exudative type (fig. 4). Within the consolidated parts of the lungs the alveolar cells frequently undergo degenerative changes characterised by nuclear pyknosis. Later the cells break down and nuclear debris can be identified amongst the alveolar contents.

Lymphocytes and plasma cells, and especially polymorpho-nuclear leucocytes, are scattered all over the lungs, particularly in their consolidated portions. They occur individually or in small groups within the thickened inter-alveolar septa and in the foci of consolidation. Monocytes, sometimes mixed with a few polymorpho-nuclears, are particularly numerous around vessels and bronchioles, where they form thick cuffs.

Lesions of the bronchioles vary. In some animals only slight congestion of the mucosa is seen; others show signs of acute catarrh with mucosal congestion and abundant secretion, sometimes containing masses of leucocytes. Occasionally desquamated alveolar cells or degenerated bronchial epithelial cells can be seen in the lumen.

Lesions of the pleura run parallel with those of the underlying lung. Over slightly inflamed non-consolidated lung the pleura appears almost normal, but over the consolidated portions the endothelial cells, considerably enlarged, show marked vacuolar degeneration of the cytoplasm, with lysis of the nucleus, which may lead to their disappearance.

### RICKETTSIÆ

Rickettsiæ are seen as fine dots, cocco-bacilli or tiny rods, varying very much in size, the dots from the extreme limit of resolution to granules easily seen under the oil-immersion objective, the cocco-bacilli from oval granules to short rods, the rods attaining a length of two microns. The extremities of both cocco-bacilli and rods are rounded. Quite often the rods are narrowed in the middle and look like tiny diplococci. Sometimes they are united to form short chains. They may contain granules of slightly greater diameter than that of the bacillus, resulting in a beaded appearance. Rickettsiæ may be seen outside or inside the cells: the extracellular and intracellular groups will be considered separately.

#### *Extracellular rickettsiæ*

These may be found individually, in clumps, in large groups or in voluminous masses. Individual rickettsiæ are best seen in alveoli or bronchioles filled with exudate, in which they occur in large numbers

(fig. 5). In other alveoli filled with inflammatory oedema the rickettsiæ are not found individually but in clumps, which may be small, composed of only a few organisms, or as large as the nucleus of an alveolar cell (fig. 6). They are irregular in shape, with projecting edges, the organisms composing them being arranged loosely or densely. Very often individual rickettsiæ and clumps coexist in the same alveolus, the former numerous in the middle, the latter disposed mainly in the neighbourhood of the alveolar wall. In heavily infected lungs the rickettsiæ form large groups (fig. 7) or even voluminous masses (fig. 8). The size and shape of these structures varies greatly, the largest being easily seen at low power magnification. The limits are either sharp (fig. 8) or ill-defined (fig. 7): in the latter case individual rickettsiæ are found at the periphery.

### *Intracellular rickettsiæ*

The intracellular rickettsiæ can be seen in several kinds of cells in the infected lung, but, since they are particularly numerous in the alveolar cells, these will be described first. In heavily infected lungs there is scarcely an alveolar cell which does not contain rickettsiæ, but the number of organisms present varies greatly. Only the cytoplasm is infected and if the organisms are few they can be distinguished as cocco-bacilli or rods identical in all respects with those described in the exudate (fig. 9, *d* and *e*). In other cells the rickettsiæ are so numerous that they almost fill the cytoplasm, while yet other cells are literally stuffed with them (fig. 9, *a*, *b* and *c*). In some cells the rickettsial masses are situated in the peripheral cytoplasm, the nucleus being pushed to one side and flattened, so that the cell takes a signet ring form.

In other cells, instead of being scattered all through the cytoplasm, the organisms develop in one or several spots, forming well defined granular structures of regular shape and sharp outline. Usually round or oval, they may be triangular, fusiform, pyriform, beaded or with lateral protuberances (fig. 10). Their size is variable, some being the size of a blood platelet, others so large that two or three of them fill the cytoplasm of the harbouring cell. In certain cells there are whole clusters of these granular structures, pressed closely against each other and only separated by fine but distinctly visible clear lines (fig. 9, *f*, *g* and *h*). The rickettsiæ composing them are so closely packed that they cannot be seen in their whole length. Nothing resembling a limiting membrane has been seen at the periphery of these granular bodies. Within non-consolidated lung, single rickettsiæ are found in or among the cells of the thickened inter-alveolar septa (fig. 11) and any existing granular bodies protrude into the lumen of the alveoli (fig. 10). Within consolidated lung, organisms lie either among the cells, singly or grouped in fine strands, or inside the cells lining the disrupted alveoli (fig. 12).

## EXPERIMENTAL TYPHUS PNEUMONIA IN MICE



FIG. 5.—Murine typhus. Numerous rickettsiae lying singly in the exudate filling the disrupted alveoli. Fine dots, coco-bacilli and tiny rods can be seen. Rickettsial staining, improved method.  $\times 1250$ .

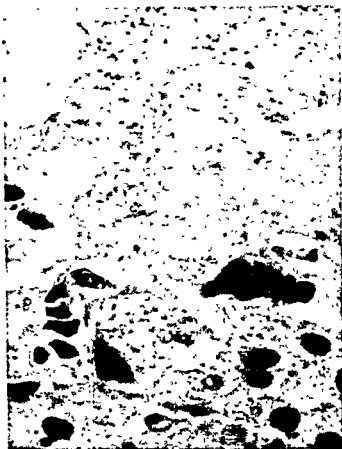


FIG. 6.—Murine typhus. Clumps of rickettsiae in the exudate filling the disrupted alveoli. In the lower part a triangular cluster and numerous rickettsiae, both extra- and intra-cellular. Same method.  $\times 1250$ .



FIG. 7.—Murine typhus. Group of rickettsiae where the organisms can be seen individually. Same method.  $\times 2100$ .



FIG. 8.—Murine typhus. Dense mass of rickettsiae in the consolidated lung. Same method.  $\times 1250$ .



## EXPERIMENTAL TYPHUS PNEUMONIA IN MICE

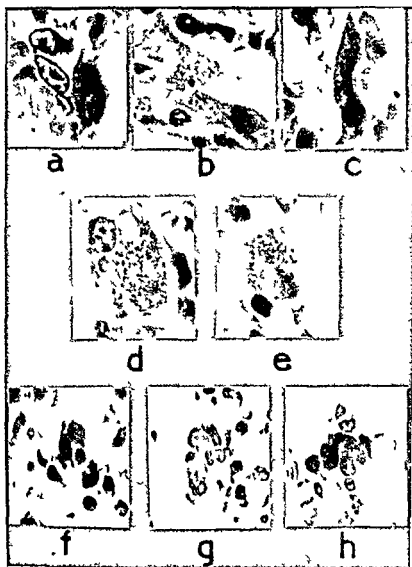


FIG. 9—(a, b and c) Murine typhus. Alveolar cells of various shapes with the cytoplasm uniformly filled with rickettsiae. (d and e) Murine typhus. Alveolar cells containing less numerous rickettsiae and showing the individual organisms. Some tiny extracellular rods beside the cell in (e). (f, g and h) Epidemic typhus. Alveolar cells packed with rickettsial masses, separated into clusters by distinctly visible clear lines. Same method. a, b, c, d, e  $\times 1200$ . f, g, h  $\times 700$ .

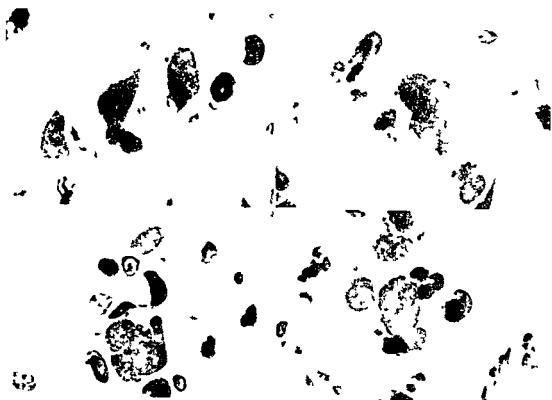


FIG. 10—Epidemic typhus. Granular bodies of various shape and size. Same method  $\times 1200$ .





The epithelial cells of the bronchial tree are the second type of cell commonly involved. In moderately infected lungs one sees scarcely a section of bronchus or bronchiole without at least one or more infected cells, sometimes in groups, while in heavily infected lungs there are bronchioles of which all the epithelial cells are filled with rickettsiæ. Typical clusters are not seen in the bronchiolar epithelium.

In striking contrast with the alveolar and bronchial epithelium, the polymorphonuclear leucocytes contain only small numbers of rickettsiæ. In the cytoplasm of most of these cells single cocco-bacilli or tiny rods can be found in varying numbers, but they are never numerous enough to fill the cytoplasm. Infected polymorphs can be found in both consolidated and non-consolidated lung, being more numerous in the former. They are seen with particular distinctness inside the bronchioles, where they may form masses filling the whole lumen.

Although most of the mesothelial cells of the pleura covering non-consolidated portions of the lung are normal, the swollen and enlarged cells covering consolidated lung are usually infected with rickettsiæ. In some, the cytoplasm is uniformly filled with organisms; in others they form round, dense granular bodies similar to those described in the alveolar cells but usually more numerous and smaller.

It is interesting to observe that the cuffing by mononuclear leucocytes of the blood vessels and bronchi already mentioned is associated with the presence of both intra- and extracellular rickettsiæ, the former lying within the alveolar cells scattered amongst the monocytes. In thin-walled vessels the rickettsial masses may lie very close to the endothelium, which, however, is seldom infected. Although in certain lungs one can see the endothelial cells of blood vessels filled with homogeneous masses of rickettsiæ or granular masses of the organism, on the whole it may be said that lesions of the vascular endothelium in infected mouse lungs are exceptional. This is in contrast with the widespread involvement of vascular endothelium in guinea-pigs described by Wolbach *et al.* (1922) following the intra-peritoneal injection of rickettsia-containing material. They concluded that the rickettsiæ of typhus had a particular affinity for the tissues of the vascular system.

On examination of lungs infected with murine or epidemic typhus one is struck by the great difference in the number of rickettsiæ present. Individual mice show much variation, but as a rule the organisms are three or four times as numerous in lungs infected with murine as in those infected with epidemic typhus. This seems to show that although they will grow in mouse lung, this forms a less favourable medium for epidemic rickettsiæ than for those of murine typhus, which are well adapted to the environment.

A point worth mentioning is that the granular bodies are larger and much more numerous in lungs infected with epidemic typhus.

This contrast is particularly striking between the epidemic strain "Algiers" and the murine strain "Port Said", both at present highly virulent. There are no noticeable differences either in the morphology of the rickettsiæ or in their behaviour in the lesions they provoke if the mice are given the same size of inoculum and if the strains compared are of equal virulence. In two series of mice infected with an equal dose of rickettsial suspension, one murine and the other epidemic, the type and extent of the lesion were the same, despite the smaller number of rickettsiæ found in the lungs of the animals infected with epidemic typhus.

## DISCUSSION

### *Nature of the inflammatory process*

By the method of infection used, the lungs are flooded with rickettsiæ and, from the start, a massive, diffuse inflammatory process involves large portions of the lungs, particularly their upper parts, in which no individual foci can be distinguished. Within the transitional area between the consolidated and non-consolidated portions, however, small foci of consolidation with numerous rickettsiæ are frequent. These spots increase in size and, reaching the consolidated area, contribute to the extension of consolidation, a feature which rickettsial bronchopneumonia has in common with bacterial bronchopneumonia.

### *Absence of secondary infection*

Although hundreds of lungs with rickettsial bronchopneumonia have been examined, in none has any histological evidence of secondary pyogenic infection been found; no cultural studies were made to confirm this finding. This is most surprising, since many of the lungs examined came from mice which had died spontaneously and in which the lungs were not fixed until several hours after death.

It is impossible to explain this phenomenon by the action of the sulphathiazole which was injected into some of the animals intraperitoneally shortly before inoculation (0.2 c.c. of a 4 per cent. suspension), since the majority of the animals had received no sulphathiazole. The more likely explanation is that the growing rickettsiæ inhibit the development of a secondary bacterial flora.

### *Morphology of rickettsiæ*

Of the four different morphological types of rickettsiæ described by Wolbach in lice (dots, cocco-bacilli, tiny rods, long threads) only the first three are commonly found in mouse lungs. Among them, rods two or three times as long as the average rods occasionally exist in lungs infected with rickettsiæ of murine type. Long threads in

## EXPERIMENTAL TYPHUS PNEUMONIA IN MICE

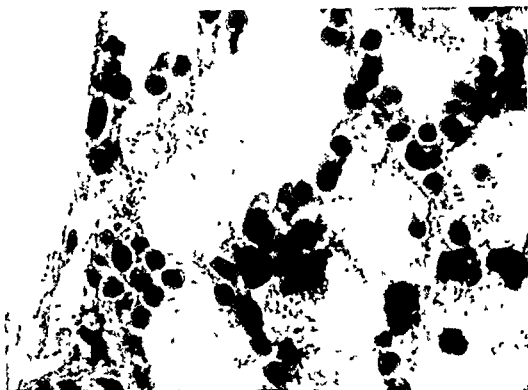


FIG. 11.—Epidemic typhus. Non-consolidated lung. Numerous rickettsiae lying singly or in masses beside and in the inter alveolar septa as well as in the alveoli of the lung. An alveolar cell packed with rickettsiae protrudes into the lumen of an alveolus. Same method  $\times 1250$ .

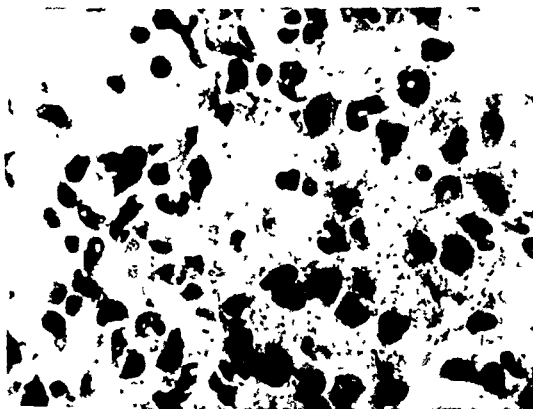


FIG. 12.—Epidemic typhus. Consolidated lung. Fairly numerous extra- and intra-cellular rickettsiae. Same method  $\times 1250$ .



great numbers both intra- and extracellular, coexisting with the usual type of rickettsiæ, have been seen only twice in lungs of mice of the same series infected with louse-borne typhus. In well stained sections the dot-shaped rickettsiæ are never seen in large masses and, if numerous, are usually associated with the other types. This shows that staining is of the utmost importance in the study of the morphology of typhus rickettsiæ and suggests that the differences in morphology between rickettsiæ in mice and those in lice—in which the large epithelial cells of the gut are stuffed with innumerable fine dots (Wolbach, Todd and Palfrey, 1922) may in fact be due to the staining technique and therefore more apparent than real.

### *Development of rickettsiæ*

It has been shown that, in mouse lung, rickettsiæ can be found both outside and inside the cell, and may occur individually or in conglomerations. This being so, the question arises whether rickettsiæ can develop only inside the cell and what is the biological significance of the conglomerations. It would seem that in mouse lungs rickettsiæ develop best inside certain cells, particularly the alveolar cells and epithelial cells of the bronchial tree. The septal cells of the alveoli as described by Gazayerli (1936) and others possess marked phagocytic powers which may well be responsible for the occurrence of rickettsiæ within them, but this mechanism cannot be invoked for the presence of equally numerous rickettsiæ in the other lining cells of the alveoli and bronchial tree. Once within the cell, the organisms are not destroyed but find therein a medium particularly favourable to their development. As for the conglomerations of rickettsiæ, both extra- and intracellular, these seem to be centres of development of the organism. It may be supposed that, increasing in size, the clumps merge into each other and give rise to groups and masses. If that hypothesis is correct then it must be admitted that typhus rickettsiæ can grow not only inside but also outside the cell.

### SUMMARY

1. An anatomical and histological study of the changes produced in the lungs of mice by the intranasal inoculation of rickettsiæ is reported. A bronchopneumonia results, consolidation being brought about either by the swelling and desquamation of alveolar cells or by inflammatory œdema filling up the alveolar spaces or by a combination of these processes. Leucocytes do not play a uniform or consistent part in the process.

2. Rickettsiæ occur as dots, cocci-bacilli or tiny rods both extra- and intracellularly. In the former situation they occur either individually or in clumps, large groups or voluminous masses; in the latter, predominantly within alveolar cells and bronchial epithelium

but also inside polymorphonuclear leucocytes and mononuclear cells. Within the alveolar cells they occur as diffuse masses or as granular structures.

3. Although the histological lesions are identical with both murine and louse-borne strains of typhus, rickettsiæ are much more abundant in lungs infected with murine strains.

4. The lack of histological evidence of secondary infection is emphasised.

5. The morphology and development of rickettsiæ are discussed and the possibility of the extracellular growth of these organisms is admitted.

I wish to express my indebtedness to the Director of Pathology, Army Medical Services, for the opportunity of working in a military laboratory, and to Professor Nowakowski and his successor, Colonel Szczodrowski of the Polish Ministry of Health, for their interest and financial help.

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## FACTORS INFLUENCING THE PRESERVATION OF STORED RED CELLS \*

J. F. LOUITT

*From the S.W. London Blood Supply Depot*

STORED blood has proved of incalculable value both for emergency and for routine transfusion purposes. In practice it is as effective as fresh blood for supplementing the deficient blood volume in cases of shock and the number of biologically functioning red cells in cases of anæmia. The vast majority of blood transfusions are given for one or other or both of these reasons. The factors which influence the length of time for which blood can usefully be stored are largely unknown. It is generally agreed, however, that thermostatically controlled refrigeration is beneficial. Increased knowledge of the effect of other physical and chemical phenomena is needed. Hitherto, these problems have been investigated almost exclusively *in vitro* by observing the effects of storage on various physical and chemical properties of the blood—the fragility of the erythrocytes to hypotonic saline, the rate of spontaneous hæmolysis and of potassium diffusion, glycolysis, phosphorolysis and so on. In the last few years, however, it has been shown by biological methods that most of the conclusions drawn from the methods of examination *in vitro* were largely invalid when applied *in vivo*.

The biological methods depend upon serological or physico-chemical differences between the transfused stored blood and the blood of the recipient. The donor blood can thus be identified in the recipient and its survival determined. Red cells of a heterologous but compatible blood group, or red cells containing radio-active iron are used as the donor blood. By these satisfactory methods limited investigations only have been made. Wiener and Schaefer (1940), Bushby *et al.* (1940), Maizels and Paterson (1940), Belk and Barnes (1941), Belk and Rosenstein (1942) and Denstedt *et al.* (1943) have reported results of red cell survival of blood stored in a small number of preservatives. Mollison and Young (1941-42), in a more extensive survey, compared the results with a larger number of preservatives. All these workers used modifications of the original Ashby (1919) technique. Ross and Chapin (1943) have reported preliminary experiments using stored blood "marked" with radio-active iron. The two methods give

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\* A report to the Medical Research Council.



but also inside polymorphonuclear leucocytes and mononuclear cells. Within the alveolar cells they occur as diffuse masses or as granular structures.

3. Although the histological lesions are identical with both murine and louse-borne strains of typhus, rickettsiae are much more abundant in lungs infected with murine strains.

4. The lack of histological evidence of secondary infection is emphasised.

5. The morphology and development of rickettsiae are discussed and the possibility of the extracellular growth of these organisms is admitted.

I wish to express my indebtedness to the Director of Pathology, Army Medical Services, for the opportunity of working in a military laboratory, and to Professor Nowakowski and his successor, Colonel Szczodrowski of the Polish Ministry of Health, for their interest and financial help.

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5 parts of citrate. In one experiment, to four such mixtures one part of 15 per cent. glucose, 15 per cent. xylose, 15 per cent. arabinose and 0.25 per cent. ascorbic acid respectively was added. The corresponding spontaneous hæmolysis figures after 28 days were 1.4, 3, 3 and 3.3 per cent. In the other experiment, to six such mixtures one part of 15 per cent. glucose, fructose, sucrose, galactose, lactose and mannitol respectively was added. The spontaneous hæmolysis figures after 28 days were 2, 2, 2.5, 3.5, 4 and 10 per cent. respectively.

## DISCUSSION

### *The methods*

*Estimation of red cell survival.* In this laboratory the modifications of the Ashby method used have given consistent results. Individual counts on the same sample agree to within  $\pm 5$  per cent. when 1000 free cells are counted. In any single case, therefore, when fresh or well preserved blood is transfused the figures obtained for survival at various intervals after transfusion, when plotted graphically against time, fall roughly on a straight line (*cf.* Brown *et al.*, 1944). Poorly preserved blood is eliminated rapidly at first. The rate of destruction is then slowed and an obtuse angled or curved graph is obtained. The reliability of the method is attributed to the use of sera with a very high titre and avidity, used at the optimal dilution recommended by Mollison (1944). The high titre sera used showed a zone phenomenon and the optimal dilution of the serum was often as high as 1 : 32. Blank counts of inagglutinable cells were frequently below 10,000 per c.mm. and always below 50,000.

The method of calculating the proportion of cells surviving is open to criticism, as the cells destroyed during the actual time of the transfusion are not estimated. The results, therefore, probably give too optimistic a figure for cell survival. Mollison and Young (1941-42), however, state that only in the case of a few of the samples of stored blood, which later underwent rapid destruction, is the concentration of donor cells appreciably lower than expected in the sample taken immediately after transfusion. Calculation of the number of cells destroyed during transfusion could have been determined directly by an accurate estimation of the recipient's plasma volume before and after transfusion and by estimating the total number of cells transfused. This, however, was not considered practicable. Alternatively, calculation of the number of cells destroyed from estimations of pigment production during transfusion was not considered feasible because of the varying rates of bilirubin excretion by the liver in different subjects. It is considered that standardisation of the type of recipient and of the amount and time of transfusion gives a fair basis for case to case and group to group comparison. This is also confirmed by the results obtained with the radio-active iron method of following red cell survival.

*Spontaneous hæmolysis test.* This crude method was considered adequate for the purpose. Spontaneous hæmolysis tests have only a negative value for determining the preservation of erythrocytes. It was used to sort out potentially good solutions from inferior ones.

### *The results*

*The effect of pH.* The findings of Loutit *et al.*, that certain citric acid-tri-sodium citrate-glucose solutions were better preservatives than comparable tri-sodium citrate-glucose solutions, suggested that

Series no.	Blood (c.c.)	Diluent (c.c.)	Solids in diluent	Days of storage	No. of cases	Plasma-diluent			Percentage survival <i>in vitro</i> (days)			
						Cit.	Na	pH range	1	7	21	
												g. per 100 c.c.
I	440*	100	Tri-sod. cit. . . . .	11-17	3	0.53	0.44	...	...	25	11	0
	430*	110	{ Tri-sod. cit. . . . . Glucose . . . . .	11-15	4	0.52	0.43	7.55	6.85	88	77	60
	420	120	{ Di-sod. cit. . . . . Glucose . . . . .	14	5	0.40	0.33	7.05	6.05	91	89	73
	Series I. An acid sodium citrate anti-coagulant without glucose is as effective as or more effective than a somewhat similar tri-sodium citrate-glucose anti-coagulant in the preservation of red cells. This tri-sodium citrate-glucose anti-coagulant had previously been shown to be markedly superior to the tri-sodium citrate without glucose.											
II	420	120	Di-sod. cit. . . . .	28	2	0.40	0.33	7.05	6.05	48	31	21
	420†	120	{ Di-sod. cit. . . . . Glucose . . . . .	28	7	0.40	0.33	7.05	6.55	81	74	59
Series II. The addition of glucose to the acid citrate anti-coagulant still further enhances its preservative powers.												
III	420	120	{ Di-sod. cit. . . . . Glucose . . . . .	28	7	0.20	0.28	7.20	6.60	85	78	62
	420†	120	{ Di-sod. cit. . . . . Glucose . . . . .	28	7	0.40	0.33	7.05	6.55	81	74	59
	420	120	{ Di-sod. cit. . . . . Glucose . . . . .	28	4	0.60	0.38	6.80	6.50	73	59	42
	420	120	{ Di-sod. cit. . . . . Glucose . . . . .	28	2	1.0	0.48	6.35	6.10	48	35	23
Series III. The acid sodium citrate-glucose was used. The blood and glucose proportions were kept constant and the acid sodium citrate concentration (and therefore also the pH) was varied. Preservation was maximal with minimal sodium and citrate radical concentration and an initial pH of the plasma-diluent of 7.20.												

IV	420	120	{Di sod cit. Glucose . Tri pot. cit Citric acid Glucose . Di sod cit Sod. chloride Glucose .	. . . . . . . . . . . . . . .	. . . . . . . . . . . . . . .	1 g. 3 g. 0 85 g. 0 27 g. 3 g. 1 g. 0 0 g. 3 g.	28	7	0 20	0 28	7 20	6 60	85	78	62	
	420	120					28	4	0 20	0 23	7 30	6 75	88	82	68	
	420	120					28	4	0 20	0 35	7 25	6 70	83	76	63	
Series IV. Acid citrate glucose solutions were used and the citrate radical, glucose and blood proportions were kept constant. The pH was thus also fairly constant. The sodium and incidentally the chloride radical concentrations were varied somewhat. Little variation in preservative power resulted.																
V	510 480 420† 360 300	30 60 120 180 240	{Di sod. cit. Glucose .	. . . . . . . . .	. . . . . . . . .	2 g. 3 g.	{28 28 28 28 28	7 5 7 5 4	0 44 0 43 0 40 0 38 0 35	0 42 0 39 0 33 0 28 0 23	7-15 7 10 7 05 6 95 6 85	6 60 6 60 6 55 6 50 6 45	92 87 81 89 76	88 85 71 83 73	74 70 59 76 55	
Series V. Acid citrate glucose solutions were used. The total amounts of both glucose and acid sodium citrate were kept constant, but the volume of anti coagulant used and therefore the volume of blood added were varied. There was no exact parallelism between the amount of dilution of the blood and the results, though if anything the less the dilution the better was the preservation.																
VI	120 120*	120 120	{Tri sod. cit. Glucose . Tri sod cit Glucose .	. . . . . . .	. . . . . . .	1 g. 3 g. 3 g. 3 g.	14 11-15	2 4	0 17 0 52	0 30 0 42	7 60 7 55	6 90 6 85	79 88	78 77	57 60	
Series VI. In contrast with series III, a tri sodium citrate-glucose diluent was used. Variation in the amount of the tri sodium citrate used made little difference to the pH of the plasma diluent mixture or to the subsequent red cell survival.																

\* Figures from Mollison and Young (1941 42)

† Figures from Loutit and Mollison (1943)

Series no.	Blood (c.c.)	Diluent (c.c.)	Solids in diluent	Days of storage	No. of cases	Plasma-diluent				Percentage survival <i>in vivo</i> (days)			
						Cit.	Na	pH range		1	7	21	
								g. per 100 c.c.	0 days				28 days
VII	420†	120	{ Tri-sod. cit. . . . . 3 g. Glucose . . . . . 3 g. }	{ 28 28 28 }	{ 5 4 3 }	0.52	0.42	7.55	6.85	45	22	10	
	360	180				0.48	0.36	7.55	6.80	52	44	27	
	270	270				0.45	0.29	7.45	6.75	55	53	35	
Series VII. This series is comparable with series V: tri-sodium citrate was used in place of acid sodium citrate. Here, however, increasing the dilution of the blood and, incidentally, slightly decreasing the pH of the plasma-diluent resulted in improved survival.													
VIII	162	378	{ Tri-sod. cit. . . . . 4.1 g. Glucose . . . . . 3 g. Tri-sod. cit. . . . . 4.1 g. Glucose . . . . . 14.6 g. }	{ 28 24-28 }	{ 3 4 }	0.61	0.27	7.45	6.80	80	70	56	
	162*	378				0.61	0.27	7.40	6.70	83	78	50	
Series VIII. Modification of the Rous-Turner anti-coagulant so that the glucose is reduced to nearly one-fifth of the original: this has little if any effect on its preservative powers.													
IX	420	120	{ Di-sod. cit. . . . . 1 g. Sod. chloride . . . . . 0.6 g. Glucose . . . . . 3 g. Di-sod. cit. . . . . 1.73 g. Sod. chloride . . . . . 0.40 g. Glucose . . . . . 3 g. Di-sod. cit. . . . . 2.59 g. Sod. chloride . . . . . 0.50 g. Glucose . . . . . 3 g. }	{ 28 28 28 }	{ 4 4 3 }	0.20	0.35	7.25	6.70	83	76	63	
	120	120				0.35	0.36	7.10	6.60	81	69	61	
	120	120				0.52	0.42	6.90	6.50	66	61	42	
Series IX. Di-sodium citrate-glucose solutions made by mixing tri-sodium citrate with hydrochloric acid appear to be as effective as those made with citric acid. The additional chloride radical does not seem to have had any deleterious effect.													

Series X. Fructose, in contrast with glucose, has little, if any, preservative powers. Dextrin when added to glucose leads to no increase in the red cell survival time.

420	120	Di sod. cit	.	.	2 g.	28	2	0.40	0.33	7.05	6.65	48	31	21
420 †	120	{Di sod. cit. Glucose .	.	.	{ 2 g. 3 g.	28	7	0.40	0.33	7.05	6.55	81	74	59
420	120	{Di sod. cit. Fructose .	.	.	{ 2 g. 3 g.	28	2	0.40	0.33	...	...	44	37	36
420	120	{Di sod. cit. Glucose . Dextrin .	.	.	{ 2 g. 3 g. 10.8 g.	28	3	0.40	0.33	..	..	77	78	67

XI

490	0	{Defibrinated blood +glucose .	.	.	{ 3.5 g.	28	1	0	0.35	..	...	11	9	...
385	110	{Defibrinated blood +sod. chloride . +glucose .	.	.	{ 0.55 g. 2.75 g.	28	1	0	0.30	7.60	6.95	25	22	..

Series XI. Defibrinated undiluted blood plus glucose is greatly inferior to citrated blood plus glucose. Defibrinated blood diluted with hypotonic saline plus glucose is only slightly better.

XII

430	110	{Tri sod. cit. Citric acid . Glucose .	.	.	{ 2.7 g. 0.3 g. 3.0 g.	27-30	3	0.55	0.41	7.20	6.65	82	78	61
420	120	{Tri sod. cit Citric acid . Glucose .	.	.	{ 0.64 g. 0.36 g. 6.0 g.	27-30	5	0.20	0.27	7.25	6.70	96	84	66

Series XII. These figures, previously published (Loutit *et al.*), are included for comparison.

\* Figures from Mollison and Young (1941-42)

† Figures from Loutit and Mollison (1943)

the change to a lower plasma  $pH$  aided preservation. The present more extensive experiments support this hypothesis. In the case of the tri-sodium citrate-glucose preservatives the improvement noted with progressive dilution (series VII) may be referable not to the dilution itself but in part to the associated slight drop in  $pH$ . Maizels (1943-44), in discussing the effect of citration of blood, has pointed out that citrate causes a chloride shift from cells to plasma by virtue of its alkalinity and of the dilution of plasma chloride. As potassium and sodium ions are initially non-penetrating, hydrogen ions accompany the chloride. This results in a slight shift of the plasma  $pH$  towards acidity. Increase of the volume of the plasma phase by water whose osmotic pressure is sustained by non-electrolyte or an electrolyte whose ions are relatively non-penetrating will thus cause penetrating anions to leave the cell with hydrogen ions and make the external phase more acid and the cells more alkaline. If the beneficial effect of a lowered  $pH$  exerts its action on the outside and not on the inside of the cells, this would explain the slightly improved preservation.

With acid diluents (series I-V, IX and XII), with one exception, the most satisfactory resulted in an initial plasma  $pH$  of 7.1-7.3. It is probable that this represents an optimal initial  $pH$ . Solutions resulting in an initial plasma  $pH$  of less than 7 were on the whole less satisfactory, though still superior to tri-sodium citrate-glucose mixtures. It is notable that acidification without the addition of glucose (series I) was as effective in improving preservation as the addition of glucose to the ordinary alkaline sodium citrate. Glucose plus acidification (series II) was, however, superior to acidification alone.

*Effect of added carbohydrate.* While there is general agreement that glucose acts as a preservative, the optimal quantities required and its mode of action have not been determined. Mollison and Young (1941-42) concluded that the effect of glucose varied with alteration in the amount and concentration of glucose that was added. This conclusion, however, was based purely on the finding of excellent preservation with the Rous-Turner solution, where the final glucose concentration in the plasma-diluent is 2.7 per cent. With smaller proportions of diluent to blood no difference in preservation could be detected with final glucose concentrations of from 0.6 to 2.2 per cent. Expt. VIII (above) shows that a modified Rous-Turner solution, with a glucose concentration in the plasma-diluent of only 0.55 per cent., was just as effective as the original with its 2.7 per cent. glucose. There is therefore no advantage in a large excess of glucose.

From the experiments *in vitro*—spontaneous hæmolysis tests—it seemed unlikely that any of the sugars tested, except fructose and sucrose, would have any beneficial effect. Mollison and Young (1941-42) have already shown from experiments *in vivo* that sucrose is without effect. Experiments in series X (above) show that fructose also has little if any real power of preservation.

Maizels and Whittaker (1940) have shown that dextrin delays

spontaneous hæmolysis *in vitro*. Preliminary experiments, however (Maizels, 1941, unpublished report to the Blood Transfusion Research Committee of the Medical Research Council), suggested that it had little effect on survival *in vivo* and Mollison and Young (1941-42) proved conclusively that dextrin alone, in spite of this effect on spontaneous hæmolysis, had little preservative effect as judged by findings *in vivo*. Experiments in series X also show that dextrin added to glucose does not further improve preservation.

The preservative effect of glucose can hardly be explained as due to its physical and chemical properties. If this had been the case, similar preservative effects might be expected from other sugars, reducing agents etc. Though the rate of glycolysis in citrate-glucose preservatives does not appear directly proportional to the degree of preservation, the beneficial effects of glucose are probably due to the fact that it can be used in red cell metabolism. That catabolism of glucose is occurring is suggested by the loss of reducing power of both the plasma-diluent and red cells during storage of blood in both di-sodium and tri-sodium citrate-glucose preservatives (Loutit *et al.*).

*Effects of varying the proportions of blood and diluent.* It is widely believed from the results of tests *in vitro* that increasing the proportion of diluent to blood causes progressive improvement in the preservation of the blood. This was not confirmed by Mollison and Young (1941-42) from investigations *in vivo*. They chose, however, as diluent isotonic tri-sodium citrate solution. The Rous-Turner solution, which is the best example of an excellent preservative with a large proportion of diluent to blood, is hypotonic (superficially the Rous-Turner solution is isotonic with plasma, but as five-sevenths of it is isotonic glucose and as glucose rapidly permeates the erythrocytes (Klinghoffer, 1940; Maizels, 1941) it virtually becomes grossly hypotonic). The present experiments were devised so that while the volume of diluent was varied the total quantity of citrate was kept constant. With tri-sodium citrate as anti-coagulant (series VII) progressive dilution did cause improved preservation, presumably, as pointed out, on account of the lowering of *pH* from the chloride shift. With the acid di-sodium citrate, on the other hand (series V), this did not obtain. Here, any chloride shift would have less effect on the *pH* of the external plasma phase, as the buffering power of citrate is greater in this *pH* range. Moreover, if the apparent optimal initial *pH* of 7.1 to 7.3 is a real factor in aiding preservation, the optimal lowering of *pH* is overstepped. In fact the most effective solution tested had a proportion of diluent to blood of 1 : 17 only. It is most unlikely, therefore, that a high diluent/blood ratio alone plays any part.

*Effect of tonicity.* It is usually recommended that diluents should be isotonic with plasma. There is no confirmation of this recommendation from the present experiments. Hitherto, the best alkaline preservatives (Rous and Turner, 1916) and acid preservatives (Murray, 1943, unpublished report to the Blood Transfusion Research Com-



mittee of the Medical Research Council) were hypotonic as judged by their citrate content and neglecting the glucose. However, a small volume hypertonic acid solution (series V) can be equally effective and with alkaline solutions hypotonic diluents were not necessarily better than comparable isotonic solutions (series VI).

*Effect of final citrate concentration.* Maizels (1943-44) has suggested that citrate, besides preventing coagulation, has another function, namely that of adding to the plasma a large amount of non-penetrating anion which acts in osmotic opposition to the non-penetrating anion present in the erythrocytes. Certainly defibrinated blood to which had been added glucose alone and glucose in 0.5 per cent. sodium chloride survived very poorly after one month's storage compared with similar citrated blood. The present range of experiments, however, does not suggest that there is any constant relationship between the preservative powers of a solution and the citrate content of the final plasma-diluent. There was a similar lack of correlation between preservative power and the initial sodium content of the plasma-diluent. The addition of sodium chloride to di-sodium citrate-glucose solutions (series IX) seemed to have no adverse effect.

It would appear that, while there is an optimum pH for red cell preservation, the other physical and chemical properties of the diluent here examined can vary within wide limits with little effect on the preservation of the erythrocytes. Preservation of the integrity of the red cell membrane and of the enzymic functions of the erythrocytes would seem to be the ideal to be aimed at and preservative solutions should be chosen with this in view.

### SUMMARY

Following transfusion of stored blood the survival of the red cells has been determined by a modification of Ashby's serological method. The preservative powers of a number of different anti-coagulant preservative solutions have been compared.

The results indicate that an initial plasma-diluent pH of from 7.1 to 7.3 is optimal and confirm that glucose has an additional preservative effect. Other physical and chemical factors examined have no constant influence on the red cell preservation.

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# FURTHER OBSERVATIONS ON THE ADENOSINE EQUIVALENT OF THE BLOOD OF RABBITS FOLLOWING LETHAL FORMS OF TISSUE INJURY

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IN a previous paper (Stoner and Green, 1944) it was shown that in the rabbit, after suspension in the head-up position ("gravity shock") and after prolonged ischaemia of the hind-limbs, there is a rise in the adenosine equivalent of whole blood. In the present paper an extension of these observations to rabbits submitted to other types of gross tissue injury, namely trauma and dehydration, is reported. We have also determined the adenosine equivalent of the blood following the administration of cyanide, since a prominent feature of shock following limb ischaemia, trauma and dehydration is a decrease in tissue respiration. Cole *et al.* (1944) and Mayerson (1944) have shown that this is also true of "gravity shock".

## METHODS

Ten rabbits were submitted to severe mechanical trauma. After two control specimens of blood had been taken with an interval of 15 mins. between them, the rabbit was anaesthetised with nembutal and given an equal number of blows to each thigh with a raw-hide mallet weighing 135 g. Further blood samples were then taken at intervals after the trauma. Ten control rabbits were treated in a similar way except that they received no trauma. The two experiments—control and trauma—were run side by side to ensure uniformity of conditions.

Dehydration "shock" was produced in 8 rabbits by the intraperitoneal injection of a 40 per cent. glucose solution (see table III). Blood samples were taken before and at intervals after the injection.

Cyanide was administered as the sodium salt to a series of 7 rabbits by a variety of routes—subcutaneous, intramuscular or intraperitoneal (see table IV). The findings in this and the dehydration group were compared with those in rabbits submitted to repeated phlebotomies (see "gravity shock" control group, Stoner and Green).

Blood samples were taken from the ear vein or by cardiac puncture. The usual practice was to bleed from the ear until the peripheral blood flow became reduced, when resort was made to cardiac puncture. Experimental and control animals were treated similarly.

The method used for the determination of the adenosine equivalent of the blood has been described previously (Drury *et al.*, 1937-38, as modified by Stoner and Green).

## RESULTS

*Traumatic shock*

The degree of trauma used led to death in all but one of the 10 rabbits (table I). The survival times varied from 20 mins. to 6 hrs. Two of 10 control animals died during the experimental period as the result of hæmopericardium.

TABLE I

*Effect of trauma on the level of the adenosine equivalent in the blood (adenosine equivalents in  $\mu\text{g. per c.c. whole blood corrected for Hb per cent.}$ ). Control values shown in columns A and B*

Rabbit no.	No. of blows to each thigh	Adenosine equivalent ( $\mu\text{g. per c.c. whole blood}$ )										
		Before trauma		After trauma (time in mins.)								
		A	B	15	30	60	90	120	210	240	270	320
44/67	25	220	330	...	400	...	320	...	410	...	380	D
44/87	40	310	340	360	D							
44/108	40	230	260	...	260	D						
44/58	50	280	270	440	D							
44/59	50	290	310	390	D							
44/65	50	200	190	...	370	...	380	D				
44/74	50	240	220	320	D							
44/93	50	300	330	360	D							
44/101	50	280	300	...	280	290	...	320	300	...	...	270*
44/115	50	260	290	370	490	D						

D = died. \* = survived.

The only abnormal post-mortem findings were at the site of the trauma. Both femora usually showed comminuted fractures, with hæmorrhage and a gelatinous exudate in the surrounding muscles. The autopsies were performed immediately after death and the traumatised muscles were judged, by clinical standards, to be non-viable. The picture was very similar to that seen in man after severe trauma to the limbs.

Hæmodilution, as judged by changes in the hæmoglobin concentration, occurred in both experimental and control animals to an equal degree.

It is seen (table I) that the adenosine equivalent of the blood rose to some degree above the mean control value in all 10 traumatised animals. The maximum rises ranged from 6 to 95 per cent. (average 39 per cent.). In the group submitted to anaesthesia together with repeated blood sampling but no trauma (table II), there was a relatively small rise in most of the rabbits, the maximum rise being 30 per cent. (average 11 per cent.). Examination of the data shows that the differences between the blood values before and after trauma are significant when compared with the corresponding values in the

anaesthetic control group Such an increase would have occurred by chance less than once in 100 experiments

TABLE II

Effect of repeated blood sampling and anaesthesia on the adenosine equivalent in the blood (adenosine equivalents in  $\mu\text{g}$  per c.c. whole blood corrected for Hb per cent) Control values before anaesthesia shown in columns A and B

Rabbit no	Adenosine equivalent ( $\mu\text{g}$ per c.c. whole blood)								
	Before anaesthesia		After anaesthesia (time in mins)						
	A	B	15	30	60	90	120	210	270
44/66	320	310		340		400			
44/68	270	250		340		330		270	270
44/70	310	310		300		310			
44/73	210	210	230			180			
44/86	330	370	340				370		
44/92	290	310		270			330		
44/100	220	210		240*					
44/103	270	260	260		230		250		
44/110	180	190		220*					
44/114	300	280	300						

\* Death from haemopericardium

### Dehydration shock

After the injection of hypertonic glucose the rabbits passed rapidly into a shock like state. A greatly increased respiratory rate and terminal convulsions were prominent clinical features. Seven of the 8 rabbits died, the majority surviving not more than 2 hours. As judged by the haemoglobin level both haemoconcentration and haemodilution occurred. The latter finding was unexpected and indicates, as would seem likely *a priori*, that the condition produced by the intraperitoneal injection of hypertonic glucose solution is not due solely to the effects of dehydration. At autopsy there was but little free fluid in the peritoneal cavity but pulmonary oedema and small pleural effusions were common. The most striking finding was the enormous dilatation of the bowel. This commenced in the distal end of the colon and often extended well up into the small intestine. Scattered submucous haemorrhages were seen in the dilated intestine. The dilatation occurred shortly before death and was usually accompanied by watery, often blood stained, diarrhoea.

The effect of this treatment on the adenosine equivalent of the blood is shown in table III. There was a definite increase in 6 of 8 rabbits, ranging from 23 to 77 per cent (average overall increase 40 per cent). It was previously found (Stoner and Green) that control rabbits bled in a similar way over a period of 4 hours showed an average rise in the blood adenosine equivalent of only 12 per cent.

Examination of the data shows that the increase in the experimental as compared with the control group would have occurred by chance less than once in 20 experiments.

TABLE III

*Effect of dehydration shock on the level of the adenosine equivalent in the blood (adenosine equivalents in  $\mu\text{g. per c.c. whole blood corrected for Hb per cent.}$ )*

Rabbit no.	Dose of 40 per cent. glucose (i.p.) (c.c. per kg. body wt.)	Adenosine equivalent ( $\mu\text{g. per c.c. whole blood}$ )						
		Initial level	After injection of glucose (time in hours)					
			1	2	3½	4	5	6
44/85	30	260	460	D				
44/89	30	280	320	390		300		
44/90	40	260	...	310	...	320	D	
44/104	40	260	250	D				
44/94	50	180	200	270	310D			
44/97	50	230	240	230D				
44/99	50	270	330	340D				
44/102	50	250	370	360D				

D = died

### Cyanide poisoning

It is difficult to express the results precisely, since the cyanide was administered by different routes and in variable dosage in an effort to prolong the symptoms of poisoning over a long period. This end was not achieved. The data show however that cyanide poisoning was, in all 7 rabbits, accompanied by an increase in the adenosine equivalent of whole blood (table IV). The individual increases were

TABLE IV

*Percentage increase in the adenosine equivalent of whole blood after varying doses of cyanide*

Rabbit no.	Body weight (kg.)	Dose of NaCN (mg.)	Route	Survival time (mins.)	Percentage increase in the adenosine equivalent of whole blood
44/91	1.5	11.0 (in divided doses)	S.C. }	>300	39
		2.0	I.P. }		
44/95	1.5	4.5	I.P. }	45	82
44/96	1.3	7.8	I.P. }	9	8
		2.6	I.P. }		
44/98	1.3	2.6	I.P.	120	24
44/105	1.2	4.5	I.M.	10	19
44/106	1.7	5.0	I.M.	>300	63
44/107	2.0	5.0	I.M.	20	23

variable but statistical analysis shows that in the group as a whole such an increase would have occurred by chance less than once in 100 experiments.

## DISCUSSION

When these results are considered with those previously reported (Stoner and Green) it becomes apparent that several different forms of bodily insult are accompanied in the rabbit by an increase in the adenosine equivalent of the blood. Although the increases found after trauma and after dehydration are not so great as those following a lethal period of ischaemia they are statistically significant and, as we have already shown, they are not primarily a result of the diminished renal function which so often follows severe tissue injury.

The fact that there is an increase in the blood adenosine equivalent in dehydration shock needs further comment, as there is no gross localised tissue damage in this condition. We had previously found (Green and Stoner, 1944) that the injection of inorganic magnesium increased the severity of this and other types of shock, including that produced by adenosine triphosphate (ATP) and various adenosine and inosine compounds. We therefore suggested that the accentuation of the effects of tissue damage by magnesium might be the result of a disturbed balance of ATP in the tissues of the injured animal. It was further suggested that in dehydration shock such a disturbed balance might result from oligaemia, which, by producing a general tissue hypo-oxygenia, renders the cell membrane more permeable to ATP. The idea received some support from the finding that magnesium accentuated the toxic effects of cyanide. In the present work we have shown that cyanide poisoning causes a rise in the adenosine equivalent of the blood. It would seem possible therefore that any severe decline in tissue oxidation leads to the release of adenosine compounds from the cell. Such a mechanism was previously suggested (Stoner and Green) to account for the rise in blood adenosine in "gravity shock". It would similarly account for the rise seen in dehydration shock and indeed in shock, however induced, at the stage when severe oligaemia is present.

An important question is whether this increase is of primary or secondary significance in the profound systemic changes following severe injury. The rises in the inorganic phosphate, potassium, lactic acid etc., of the blood are probably due to the release of these substances from the damaged tissue and it would seem likely that the increase in adenosine compounds occurring shortly after injury is due to the same cause. There is the difference, however, that whilst the former compounds do not, on injection, produce a typical shock-like state, adenosine compounds, particularly the triphosphate (ATP), do so. Adenosine itself has this action, though its potency in this respect is only about one-third that of ATP. Moreover it has been shown by us (in unpublished work) that the fatal shock-like state produced in the rabbit by the intramuscular or intraperitoneal injection of ATP is associated with a roughly similar rise in the blood



adenosine to that found after lethal forms of tissue trauma. All the indications are that extracellular ATP is rapidly broken down by the tissues. Therefore either the relatively minute amounts of the unchanged compound entering the blood stream are capable of profound biological effects or these effects are initiated in some way by some chemical intermediary resulting from the rapid breakdown of ATP. Such a rapid breakdown is known to occur in one form of severe tissue injury, namely limb ischaemia (Green, 1943; Bollman and Flock, 1944). The relatively small rises in the adenosine equivalent of the blood following severe tissue injury may be indicative of a small overflow of adenosine or adenosine compounds which have escaped deamination prior to their absorption into the blood stream. This, we postulate, may be associated with the breakdown of relatively enormous amounts of ATP in the tissues, both at the site of injury and later, with progressive anoxia, over a much wider area.

In what form the adenosine responsible for the rise in the adenosine equivalent exists in the blood of the injured rabbit is not yet known. It is however unlikely, even assuming that it reaches the blood as ATP, that it remains intact as such, for dephosphorylation by blood enzymes is likely to be proceeding. In that case the tri-, di- and monophosphates and adenosine itself would presumably all contribute to the increase in the total adenosine equivalent. The amounts involved are obviously small and only isolation experiments on a large scale can finally settle the point. Whatever its chemical composition, however, provided it did not materially differ qualitatively and quantitatively from that found in the blood of rabbits killed by the injection of ATP, the hypothesis that the release of ATP from injured tissues might account for part of the systemic manifestations of severe injury would not be upset.

The term shock has been sparingly used because it is realised that, whilst the systemic reactions to many different forms of tissue injury may prove to be identical, the fact that a few symptoms and signs are common to each obviously does not establish such an identity. If ultimately it is possible to show that all the main reactions included in the shock syndrome are mediated by one common factor, whatever the immediate cause of the injury, then the word shock can be safely used on an aetiological basis. Proof is still lacking of any one such common factor. The histamine hypothesis has not stood the test of time and the ATP hypothesis still remains unproved.

#### SUMMARY

1. In rabbits a significant rise in the adenosine equivalent of the blood follows both a lethal degree of trauma to the hind limbs and a lethal degree of dehydration by hypertonic glucose solutions.
2. Fatal poisoning with inorganic cyanide also produces a significant rise in the adenosine equivalent of the blood. This gives further

support to the hypothesis that a decrease in tissue oxidation leads to the release of adenosine compounds from the cells.

3. The possibility is discussed that a release of adenosine triphosphate from the injured tissue itself, followed later by a more general release from anoxic tissue, is responsible for some of the systemic effects of local tissue damage.

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#### SUMMARY

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GROSS DEFECTS IN THE MUSCULAR AND ELASTIC  
COATS OF THE LARGER CEREBRAL ARTERIES

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(PLATES XLII-XLIV)

LOCALISED defects in the walls of the larger cerebral arteries are of interest chiefly because they may be concerned in the pathogenesis of non-inflammatory aneurysms. These usually take the form of thin-walled sacs opening into the vascular lumen through relatively narrow necks and hence they probably owe their origin to serious circumscribed deficiencies in the arterial wall and not to diffuse destructive processes. But since the mouth of the sac, though small, is yet large enough to be readily visible to the naked eye, it does not appear likely that an aneurysm can arise in a mural defect of minimal dimensions. In the present study attention has therefore been concentrated upon the larger lesions, since these are more likely to produce significant effects.

*I. Developmental defects in the muscular coat*

The frequent occurrence of inborn defects in the walls of the cerebral vessels was first demonstrated by Forbus (1928-29, 1930), who found areas of complete deficiency in the muscular coat of the arteries in about 75 per cent. of a group of over 30 normal brains. The defects were all situated at the main arterial junctions or bifurcations or at the mouths of branches, usually in the angle between the divergent channels; they were not peculiar to adults but were also found in children, including several babies under 12 months and one still-born infant; there was no scarring or other evidence of disease, whether active or obsolete, in the region of the gaps. For these reasons Forbus suggested that the defects were due to a fault in the early development of the vessels, most probably to a failure of fusion between the muscular coats of the trunk and branches, which are formed independently of each other. He looked upon these defects as the primary causal factor in the genesis of most intracranial aneurysms.

These observations were confirmed, in almost all essential particulars, by Voncken (1931), Kahlau (1937-38) and Schmidt (1937-38), who accepted Forbus's estimate of the origin and importance of the defects; by Tuthill (1933), who thought they were artefacts, and by Glynn (1940), who disputed their developmental origin and questioned their relationship to aneurysms.

Most of these defects are apparently so small that they can be seen only under the microscope, but in the present study the minor lesions have been neglected and the major deficiencies singled out by the following method.

Preparations were made from the regions of the principal junctions and bifurcations of the cerebral arteries of 40 subjects, 5 from each decade up to the eighth. Cases with meningitis or advanced cerebral atheroma were rejected but otherwise no selection was exercised. A complete set of preparations should have included both internal carotid bifurcations, the main bifurcation of each middle cerebral artery, a single H-shaped preparation of the anterior communicating artery together with the adjacent parts of the anterior cerebral vessels, and the upper and lower ends of the basilar artery, but many specimens were damaged during the manipulations and had to be discarded. The vessels were slit open with scissors, flattened out on sheets of filter paper, fixed in formol-sublimate, stained overnight in a slowly-acting alum hæmatoxylin and then washed for 24 hours in running water. The preparations, on emerging from the wash water, were deep blue in colour, with a shiny inner surface and a whitish woolly zone of unstained collagen on the exterior. Complete muscular defects appeared as small areas of intense pallor, most clearly seen when the specimens were viewed by transmitted light, and in many specimens lightly stained areas of partial deficiency were also apparent. Small rectangular pieces containing the defects were cut from the preparations, dehydrated in alcohol, cleared in clove oil (which seemed to make the tissue rather less brittle than the usual clearing agents) and embedded in paraffin with the long axis of the defect lying, as nearly as possible, either parallel with or perpendicular to the edge of the block. Serial sections were attempted in all cases but the tissue was always very fragile, probably as a result of its long immersion in the acid alum solution, and the sections were often unfit for measurement and served only for the identification of the lesion.

A considerable number of the pale areas seen in the gross specimens proved to be merely traumatic artefacts, but in the sections these were readily distinguished from the natural lesions by the broken edges of the gap in the muscle. Others were foci of advanced medial degeneration and fibrosis. The remainder had the characters of the developmental defects described by Forbus.

Gross developmental defects in the muscular coat were demonstrated in 27 of the 40 sets of cerebral vessels examined, 48 defects in all in 201 preparations from the main vascular junctions and bifurcations. Details of their distribution are given in table I, which shows that they were all situated on arteries of the carotid system and chiefly on the middle cerebral vessels; there were none on the basilar arteries. A few additional foci were identified at the mouths of minor branches but none in other parts of the vessels. Many patches of well marked hypoplasia of the muscularis were also found at the arterial bifurcations, the largest being situated at the upper end of the basilar artery; in these foci the muscular coat sometimes consists of only one or two layers of cells and they are obviously a less extreme variant of the condition under consideration (fig. 6).

In naked-eye preparations of stained vessels foci of total muscular deficiency appear as patches of intense pallor, roughly elliptical in shape. The area of the focus, as is apparent from the measurements recorded in table II, is usually well under 1 sq. mm. although it

TABLE I

*Gross developmental defects in the muscular coat of the cerebral arteries  
age distribution and location*

Age	Internal carotid (bifurcation)				Middle cerebral (bifurcation)				Anterior com municans		Basilar artery				Totals	
	Right		Left		Right		Left				Upper end		Lower end			
	S	D	S	D	S	D	S	D	S	D	S	D	S	D		
0 9	5	0	4	0	4	1	3	2	2	0	4	0	2	0	24	3
10 19	4	0	2	0	3	1	3	0	3	0	4	0	3	0	22	1
20 29	4	0	5	0	5	4	5	2	5	1	3	0	3	0	30	7
30 39	4	0	4	0	5	2	5	3	3	0	3	0	3	0	27	5
40 49	3	0	5	2	4	2	5	3	4	1	4	0	2	0	27	8
50 59	3	0	4	1	2	2	5	5	4	0	4	0	4	0	26	8
60 69	2	0	2	0	5	3	3	3	3	1	4	0	2	0	21	7
70 79	3	1	4	1	4	2	4	4	2	1	4	0	3	0	24	9
Totals	28	1	30	4	32	17	33	22	26	4	30	0	22	0	201	48

S = specimens examined

D = defects found

TABLE II

*Dimensions of developmental defects in the muscular coat  
of cerebral arteries*

Site of defect	Age of subject (years)	Dimensions of defect (in mm)
Right internal carotid bifurcation	73	1.96 × 0.61 +
Left " " "	49	1.60 × 0.56
	10	1.10 × 0.30
	22	1.40 × 0.38
	27	1.28 × 0.27
	31	0.96 + × 0.64
Right main middle cerebral bifurcation	43	2.14 × 0.75 +
	62	1.72 + × 0.52 +
	63	1.96 + × 1.40
	65	1.04 × 0.92
	73	0.93 + × 0.90
	78	2.00 + × 0.79
	27	1.34 + × 0.35
	49	1.10 × 0.70 +
	52	1.39 + × 0.69
	54	1.00 × 0.48 +
Left main middle cerebral bifurcation	56	0.85 + × 0.84
	71	1.04 × 0.48
	73	1.20 × 0.78 +
	75	1.90 + × 0.92
	78	2.22 + × 0.58
Anterior communicating region	63	2.34 × 0.95 +
	78	0.64 × 0.35

Many defects were unavoidably cut in opening the vessels but when only a small portion appeared to be missing the dimensions are given with a + sign

Most of these defects are apparently so small that they can be seen only under the microscope, but in the present study the minor lesions have been neglected and the major deficiencies singled out by the following method.

Preparations were made from the regions of the principal junctions and bifurcations of the cerebral arteries of 40 subjects, 5 from each decade up to the eighth. Cases with meningitis or advanced cerebral atheroma were rejected but otherwise no selection was exercised. A complete set of preparations should have included both internal carotid bifurcations, the main bifurcation of each middle cerebral artery, a single H-shaped preparation of the anterior communicating artery together with the adjacent parts of the anterior cerebral vessels, and the upper and lower ends of the basilar artery, but many specimens were damaged during the manipulations and had to be discarded. The vessels were slit open with scissors, flattened out on sheets of filter paper, fixed in formol-sublimat, stained overnight in a slowly-acting alum hæmatoxylin and then washed for 24 hours in running water. The preparations, on emerging from the wash water, were deep blue in colour, with a shiny inner surface and a whitish woolly zone of unstained collagen on the exterior. Complete muscular defects appeared as small areas of intense pallor, most clearly seen when the specimens were viewed by transmitted light, and in many specimens lightly stained areas of partial deficiency were also apparent. Small rectangular pieces containing the defects were cut from the preparations, dehydrated in alcohol, cleared in clove oil (which seemed to make the tissue rather less brittle than the usual clearing agents) and embedded in paraffin with the long axis of the defect lying, as nearly as possible, either parallel with or perpendicular to the edge of the block. Serial sections were attempted in all cases but the tissue was always very fragile, probably as a result of its long immersion in the acid alum solution, and the sections were often unfit for measurement and served only for the identification of the lesion.

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LOCALISED DEFECTS IN CEREBRAL ARTERIES



FIG. 1.—Large developmental defect in the muscular coat of the anterior communicating artery. Male, aged 63.  $\times 60$ .



FIG. 2.—Large developmental defect in the muscular coat at the bifurcation of the right middle cerebral artery. Female, aged 22.  $\times 60$ .





## LOCALISED DEFECTS IN CEREBRAL ARTERIES



FIG. 3.—Medial defect due to degeneration and fibrosis of the muscular coat on one side of a minute developmental gap at the bifurcation of the left middle cerebral artery. Male, aged 75.  $\times 60$

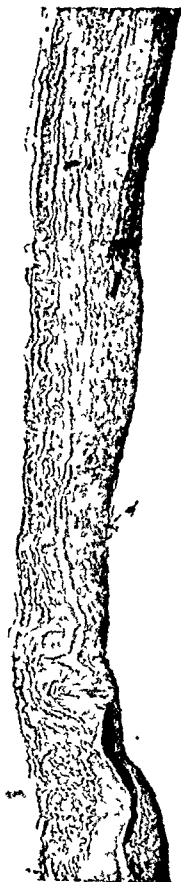


FIG. 4.—Focal erosion of the internal elastic lamina at the upper end of the basilar artery. Male, aged 59.  $\times 60$



half the thickness of the normal media and this preserved the identity of the middle coat as a separate layer. Disappearance of the muscle from the whole thickness of the wall was noted in only one or two small foci located at the bifurcations, but here the degeneration had apparently attacked hypoplastic portions of the wall, as the fibrotic media was unusually thin. Another effect, more frequently noted, was the destruction by medial degeneration of portions of the thin muscular wedge around developmental defects, sometimes leading to slight extension of the gap, but only rarely (as in the specimen illustrated in fig. 3) to a substantial increase in its size.

On the whole it appears that medial degeneration of this type is not, in itself, a common cause of total defects in the muscular coat, although it may complete or enlarge defects due to other causes.

### III. Defects in the internal elastic lamina

No systematic search was made for defects in the elastic lamina but minute erosions in the unsplit membrane were encountered in several specimens and one large defect was found by chance in a fragment of tissue from the upper end of a basilar artery. This big C-shaped gap was too irregular for exact measurement but a patch of membrane roughly 2 sq. mm. in area had been almost completely destroyed, leaving only a thin sheet of elastic tissue at the surface of the intima. Although a portion of the underlying media was fibrotic, there was no aneurysmal bulging (fig. 4).

### IV. Defects in the muscular and elastic coats of atheromatous vessels

In patches of advanced atheroma the internal elastic lamina is invariably split into several layers, the stoutest zone usually lying deep to the intimal plaque while the others traverse its substance. As the disease advances much of the elastic tissue degenerates, the frailer strands usually disintegrating first while the thicker laminæ may long remain intact or show only minor erosions. But occasionally all the main elastic layers disintegrate in an area of considerable size, leaving this part of the wall virtually devoid of elastic tissue. The degenerative process may also encroach upon the muscular coat, destroying the muscle cells in the infiltrated areas, and although such penetration is usually very limited in its scope the muscularis is sometimes widely disrupted or even completely breached by the outward extension of the lesion. Other defects in the muscle arise from so-called pressure atrophy, a process involving piecemeal degeneration of the muscle fibres, accompanied by fibrous scarring in the media.

In order to assess the frequency of these occurrences sections were made from 200 patches of atheroma in cerebral vessels over 1 mm. in diameter, a small group of contiguous sections being taken at random from each plaque. More extensive lesions would doubtless

have been found in serial sections but even the limited examination actually made shows that big defects in the muscular and elastic coats are a fairly frequent effect of atheroma. Defects involving all layers of the elastica were found in 54 patches, 13 situated at or near the edge of the atheromatous plaque and 41 in the central region. Most of the gaps were very small, but 11 exceeded 0.5 mm. in width and 5 of these were over 1 mm., including 3 over 2 mm.; the largest measured 2.5 mm. Total defects in the muscular coat were found in 26 plaques, 5 at the periphery and 21 near the centre of the patch. Their width was over 0.5 mm. in 7 instances and more than 1 mm. in 2, the largest measuring 1.6 mm. In 5 vessels there were gaps in both muscular and elastic coats in the same part of the wall, including one instance in which an elastic gap 2.5 mm. in width overlay a muscular defect of 1.4 mm.; none of these areas showed any aneurysmal bulging.

### *Commentary*

If the mere existence of a minute developmental gap in the muscular coat of an artery were enough to determine the formation of an aneurysm, almost every circle of Willis would bear a generous crop of tiny aneurysmal sacs and most of us would die from meningeal hæmorrhage in childhood or early youth. But, while medial defects are extremely common, cerebral aneurysms are relatively rare, presumably because the elastic tissue, which in these vessels is almost wholly concentrated in the internal elastic membrane, offers an effective barrier to unlimited expansion. Forbus himself believes that the disintegration of this membrane where it covers the muscular gap is an essential step in the formation of an aneurysm, and it is a pity that neither the supporters nor the opponents of his views give any consideration to this aspect of his thesis. Glynn, indeed, suggests that the strength of the vessel resides entirely in the elastica and that a gap in the muscle does not constitute a locus minoris resistentie. But, as fig. 5 shows, the wall bulges at the site of a big muscular defect even under a pressure equivalent to little more than 20 mm. of mercury, showing that there is some impairment of resistance. And the examples of focal erosion of the internal elastic membrane described above show that the muscular coat by itself can support the intravascular pressure. Both layers evidently display considerable resistance and either layer alone can prevent undue extension of the lumen. Further, it has been shown that in atheromatous vessels both elastic and muscular layers may be deficient and still no aneurysmal sac develop, the dense fibrous tissue of the intimal plaque being apparently sufficient to resist excessive distension. The wall does not yield if the outer coat is supplemented by one substantial inner layer, whether this be composed of muscle, elastic tissue or fibrous intima. This seems to be true at least in the case of the circumferential deficiencies with which we are now concerned; it may not hold for

## LOCALISED DEFECTS IN CEREBRAL ARTERIES



FIG 5—Marked bulging of a large developmental defect at the bifurcation of the left middle cerebral artery Male, aged 57 Vessels fixed by perfusion with formalin at a pressure of about 30 cm of water  $\times 60$



FIG 6—Extreme hypoplasia of the muscular coat at the upper end of the basilar artery Female aged 65  $\times 60$



more extensive lesions. Not one of these common conditions, by itself and in its ordinary form, provides a sufficient basis for the formation of a saccular cerebral aneurysm. The cause of these aneurysms must therefore be sought in some specialised lesion or in some peculiar combination of the commoner lesions.

### Summary

Circumscribed areas of complete deficiency in the muscular coats of the larger cerebral arteries are of frequent occurrence and may be due to defective development, to degeneration and fibrosis of the media or to the effects of advanced atheroma. Similar lesions in the internal elastic lamina may result from focal erosion or from atheromatous degeneration. These defects are briefly described, measurements of some of the larger examples are recorded and their possible relationship to non-inflammatory saccular aneurysms is discussed.

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# THE RELATIVE EFFECTS OF DIETARY CONSTITUENTS AND OTHER FACTORS UPON CALCULUS FORMATION AND GINGIVAL DISEASE IN THE FERRET

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(PLATES XLV AND XLVI)

A PRELIMINARY account has already been given of a form of gingival disease common in laboratory ferrets, based upon a study of the changes in the parodontal tissues as health gives way to disease (King, 1944). It was shown that the gum lesions first arose in the carnassial regions of the mouth in the neighbourhood of the openings of the salivary ducts and were accompanied by deposition of salivary calculus (tartar) on the adjacent teeth. As the disease progressed the tartar accretions increased in amount and extent and the gingivæ became more swollen and hæmorrhagic. The later phases included gingival atrophy, resorption of the alveolar bone, sometimes "erosion" of the cementum or the dentine of the tooth-roots or both, parodontal abscesses and loosening of the teeth. An intimate association was found to exist between the condition of the epithelial papillæ of the gum and the vessels running between them, this being demonstrated by slit-lamp microscopy as well as histologically.

In the present communication it is shown that this form of disease in ferrets does in fact arise as a direct result of the deposition of tartar upon the teeth. The gingival lesions are the outcome of injury of the gum by the tartar accretions. A suspicion that diet played a part in the production of these lesions arose concurrently with wartime modifications of the animals' rations, including restriction of the meat supply and less frequent access to bone-containing carcasses. It was noted by one of us (J. D. K.) that ferrets receiving these restricted rations showed varying degrees of tartar formation and parodontal disease. This and other observations referred to in the earlier paper led to a more intensive study of diet in relation to the parodontal tissues which forms the basis of the present report.

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\* Beit Memorial Research Fellow during the period of this investigation.

## MATERIAL AND METHODS

More than 130 ferrets fed on various experimental rations comprised the material for these studies, which covered a period of over two years; in addition some 80 stock animals were used for supplementary observations. Included among both groups are some of the ferrets referred to in the previous communication.

*Age, weight and sex of ferrets.* The ages of the animals at the beginning of each experiment varied according to the objects in view. At first the initial age was 6 weeks (just after weaning). Later, when the tartar-preventive factor was suspected, older animals (up to 3 months) were used, but in each experiment or comparable groups of experiments the initial ages were identical; and in this case they were first given a pre-experimental stock diet plus bone for 1-4 weeks. In most experiments the ferrets were divided into pairs, one pair to a cage, the average weight of each pair being approximately similar. A check on the general health and rate of growth was at first kept by weighing the animals twice weekly; later the weighings were less frequent. Males were preferred to females to avoid complications connected with ovulation.

*Feeding.* Cereals (when given), whole milk or milk powder, and salt mixture were first mixed with sufficient water to make a stiff lumpy paste, to which were added the other ingredients. When bread was given, only the crumb was used and this was soaked in water for 1-2 hours before use. Meat, except when autoclaved, was cut into small cubes or minced and, with cod-liver oil and food yeast (*Torula utilis*), then added to the diet. Before their effects were ascertained, nicotinamide and ascorbic acid were given directly by pipette, the former in aqueous and the latter in 5 per cent. citric acid solution; later these supplements were mixed with the main diet. Cooked meat comprised meat treated in an autoclave for 30 minutes at 15 lb. pressure. Additions of bone were made in several forms: (1) short 3-inch lengths of horse rib, with periosteal, muscular and tendinous attachments *in situ*; (2) similar lengths of rib-bone scraped free of periosteum and other attachments; (3) as (1) but crushed into a fine powder; (4) as (1) but macerated by steam into a soft pulpy mass; (5) commercial feeding bone-meal. Red marrow from ribs and yellow marrow from femur shafts were also used for tests. Liver supplements were supplied from fresh horse's liver. For testing the effects of altering the pH of the drinking water, citric acid was added to depress the pH to 4.5, ammonium chloride and sodium bicarbonate to raise the pH to 7.4.

All animals were examined before and once weekly throughout the experimental period, due attention being paid to their activity and general appearance and to the condition of their eyes, muzzles, coats, stools etc., as well as to their mouths. At various times material was collected from the gums for smear examination and culture, and latterly the gingivæ were examined *in vivo* with a specially designed capillary (slit-lamp) microscope under nembutal anaesthesia. At the end of each experiment a full post-mortem examination was made and material removed for histological study, routine staining of the parodontal tissues being supplemented by the sodium nitroprusside-benzidine technique of Pickworth (1934-35) and other methods (*vide* King). Other special experimental procedures are described below.

## RESULTS

Animals fed for periods of up to eight months on the three basal diets employed here showed a generally satisfactory rate of growth, at least when the rations were supplemented by adequate amounts of cod-liver oil and food yeast. Previous experience had indicated

that a minimum of 5-15 g. of raw meat daily, according to the age of the animal, was of benefit and this was confirmed. The addition of ascorbic acid appeared to have little or no effect and it seems probable that ferrets, like dogs, are able to synthesise sufficient

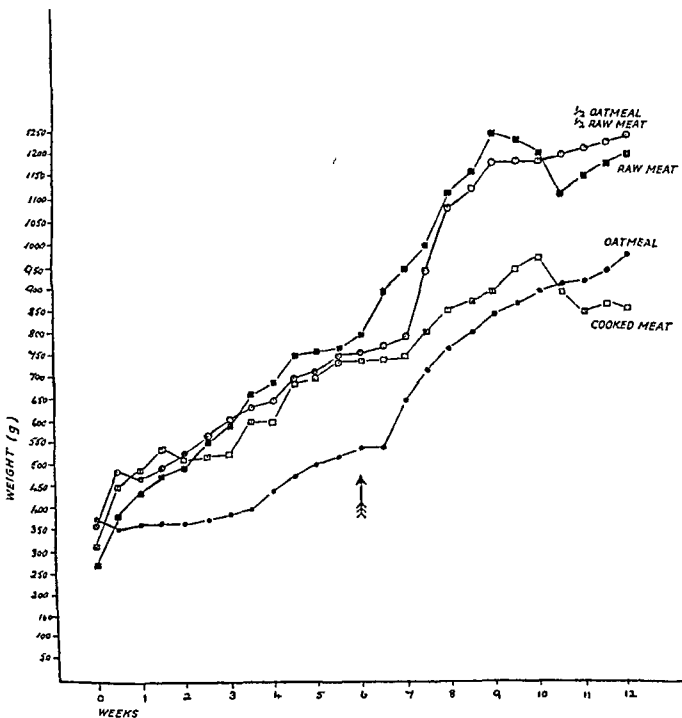


FIG. 1.—Representative weight curves of animals receiving various basal diets. Male ferrets; age at beginning of experiment 6 weeks. Time of year, May to August. Arrow indicates increase of total food from 54.5 to 94.5 g. dry weight.

quantities of vitamin C. There was an indication that the animals preferred bread to oatmeal and this was often reflected in a better initial appetite and rate of growth. As they became accustomed to the oatmeal, however, satisfactory weights were maintained (fig. 1). No significant difference in growth rate could be observed between ferrets receiving fresh milk and those given whole milk powder, at

least in the presence of adequate cod-liver oil. The general health of the animals was little affected by any of the dietary variations described and this was confirmed *post mortem*.

*Effect of diet in the production and prevention of gum disease*

Three basal diets were used in which oatmeal or bread or meat formed the chief ingredient. The relative effects on the ferrets' teeth and parodontal tissues of these diets and their various supplements are shown in tables I-III.

TABLE I

*Effect of oatmeal as main dietary ingredient on tartar formation and gingival disease in ferrets*

Dietary additions or alterations (per animal per day)	No. of animals	Exptl. period (months)	Oral conditions	
			Tartar	Gum disease
Addition of				
cod-liver oil (2.3 per cent.) . . . . .	31*	3.5	+	+
food yeast (0.5-1 per cent.) . . . . .	31*	3.5	+	+
nicotinamide (10 mg.) . . . . .	5	3.5	+	+
ascorbic acid (1.7-10 mg.) . . . . .	31*	3.5	+	+
dog-biscuit (hard lumps, 7 per cent.) . . . . .	2	3	Slight	Slight
dog-biscuit (finely powdered, 7 per cent.) . . . . .	2	3	+	+
piece of horse's rib-bone plus attachments (about 15 g.) . . . . .	6	5	—	—
piece of horse's rib-bone less attachments (about 15 g.) . . . . .	4	3.5	Slight	Slight
periosteum from 15 g. of rib-bone . . . . .	2	3	+	+
red marrow from 15 g. of rib-bone . . . . .	2	3	+	+
yellow marrow from 15 g. of femur shaft . . . . .	2	3	+	+
fresh horse liver (6 per cent.) . . . . .	2	3	+	+
Oatmeal reduced and meat increased to produce equal parts of each by dry weight	5	3.5	+	+

Total animals used . . . . . 43

Basal diet per animal per day—

Oatmeal (medium ground) . . . . . 45.75 g.

Whole milk powder . . . . . 3.5 g.

Lean meat . . . . . 5-15 g.

Salt mixture (McCollum 185) . . . . . 1 g.

\* This figure includes some of the animals used for testing the effects of the other dietary factors cited.

With *oatmeal* as the main constituent (table I) only 6 out of 43 animals in this group remained free from tartar and gum disease, and all of these six received supplements in the form of pieces of rib-bone plus periosteal, muscular and tendinous attachments throughout the experimental period of 5 months. The ferrets given rib-bone stripped of attachments (4 animals) or hard lumps of dog-biscuit (2 animals) showed relatively light deposits of tartar and their gingivae were but slightly affected. The remaining 31 animals all had heavy tartar accretions which were associated with severe gum lesions.

The addition to the basal diet of cod-liver oil, food yeast, nicotinamide, ascorbic acid, finely powdered dog-biscuit, periosteum, red bone-marrow, yellow bone-marrow, or fresh liver was without effect in preventing these conditions; nicotinamide and food yeast, however, had a slightly retarding influence on the progress of the gum lesions.

When bread (national) formed the chief ingredient (table II) 10 out of 36 ferrets remained free from disease throughout experimental periods of 5-8 months. Again all of these disease-free animals had their basal diet supplemented by rib-bone plus attachments. The 2 animals receiving additions of coarsely broken dog-biscuit showed some tartar deposition and slight gum disease within 3 months. Such conditions were not prevented by supplements of cod-liver oil, food

TABLE II

*Effect of bread (national) as main dietary ingredient on the ferret disease*

Dietary additions or alterations (per animal per day)	No of animals	Exptl period (months)	Oral conditions	
			Tartar	Gum disease
Addition of				
cod liver oil (2.3 per cent)	24*	3.5	+	+
food yeast (0.5-1 per cent.)	24*	3.5	+	+
nicotinamide (10 mg)	2	3	+	+
ascorbic acid (1.7-10 mg)	18*	3.5	+	+
dog biscuit (hard lumps, 7 per cent)	2	3	Slight	Slight
dog-biscuit (finely powdered, 7 per cent)	2	2	+	+
piece of rib-bone plus attachments (about 15 g)	10	5.8	—	—
finely crushed rib-bone and attachments (15 g)	2	3	+	+
macerated rib-bone and attachments (15 g)	2	3	+	+
Substitution of				
whole fresh milk for milk powder (5 g)	11	3.5	+	+
1 per cent. citric acid for drinking water	2	3	+	+
1 per cent. ammonium chloride (20 parts) plus 1 per cent. sodium bicarbonate (1 part) for drinking water	2	3	+	+

Total animals used, 36.

Basal diet per animal per day: bread (crumb) substituted for oatmeal in diet 1.

\* This figure includes some of the animals used for testing the effects of the other dietary factors cited

yeast, nicotinamide, ascorbic acid, finely powdered dog-biscuit, or finely crushed or macerated rib-bone plus attachments. Again there was an indication that nicotinamide, and to a lesser degree food yeast, retarded the progress of the gingival lesions in some instances. Alteration of the pH of the drinking water was also without effect, as was the substitution of fresh milk for milk powder.

Replacement of the cereal by an approximately equivalent dry weight of lean meat (in addition to the meat of the basal ration) failed to prevent the development of tartar or gum disease, except in the 4 (out of 26) ferrets receiving rib-bone plus attachments (table III).

The addition of cod-liver oil, food yeast, ascorbic acid, commercial bone meal or red bone marrow was also ineffective.

As regards pathology, the changes in the gums were not only associated with deposits of tartar upon the teeth but were, in fact, directly caused by trauma of the gingivæ by these calcareous accretions. Figs. 2-4 show the manner in which tartar deposits gradually extend from the crown of the tooth until they impinge upon and

TABLE III

*Effect of meat (lean) as main dietary ingredient on the ferret disease*

Dietary additions or alterations (per animal per day)	No. of animals	Exptl. period (months)	Oral conditions	
			Tartar	Gum disease
Addition of				
cod-liver oil (2.3 per cent.) . . . .	16*	3	+	+
food yeast (0.5-1 per cent.) . . . .	16*	3	+	+
ascorbic acid (1.7 mg.) . . . .	16*	3	+	+
piece of rib-bone plus attachments (about 15 g.)	4	3	—	—
commercial bone meal (15 g.) . . . .	2	3	+	+
red marrow from 15 g. of rib-bone . .	2	3	+	+
Autoclaved meat . . . .	6	3	+	+

Total animals used, 26.

Basal diet per animal per day: meat substituted for cereal in amounts (dry weight) equivalent to cereal dry weight in addition to the basal 5-15 g.

\* This figure includes some of the animals used for testing the effects of the other dietary factors cited.

eventually penetrate the gingival crest. Further progress of the disease is probably aided by secondary bacterial action until the alveolar bone, cementum, dentine and the mucous membrane of the cheek become involved (King). Figs. 5-9 illustrate, by slit-lamp photomicrographs of the marginal blood vessels of the gum, the dependence of the ferret's parodontal health upon adequate mechanical friction of the tooth surface. When the animal gnaws short lengths of bone plus attachments the vascular and other tissues remain normal (fig. 5), but when the bone plus attachments is given in a crushed form severe disease soon follows (fig. 7). Coarse lumps of hard dog-biscuit exert a certain amount of protection (fig. 6) but disease eventually supervenes. If the biscuit is finely powdered the lesions are much more rapid and severe (fig. 8). There is some evidence that the lesions develop more rapidly with oatmeal than with bread (fig. 9). It is significant that in the ferrets kept free from tartar and gum disease by supplements of bone plus attachments, the bone was by no means completely eaten; indeed, little more than the small amount of attached meat and some of the periosteum were ingested, while these attachments, given separately to other animals, were without prophylactic action.

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## PLATE XLV

FIGS. 2-4.—Production of gum disease in ferrets by tartar deposits. Photomicrographs of decalcified bucco-palatal sections through upper carnassial region. Haematoxylin and eosin.  $\times 60$ .

FIG. 2.—Normal appearance of gingival tissues.

FIG. 3.—Tartar deposition in buccal tooth groove just above dental margin of gum. Only the tip of the gingival crest is beginning to lose its keratinous covering, but even at this stage slight alterations of the marginal capillary pattern can be detected with the slit-lamp microscope.

FIG. 4.—Penetration of gingival crest by tartar deposits, with resulting loss of its keratinous covering, hyperplasia of the subjacent gingival epithelium, marked dilatation of the blood vessels and cellular infiltration of the neighbouring corium. All these conditions contribute to the mushroom-like eversion of the gum.

B.V. = blood vessels  
Co. = corium  
K. = keratinous layer  
D. = dentine

E.C. = enamel cuticle (Nasmyth's membrane)  
G.Ep. = gingival epithelium  
S.Ep. = sub-gingival epithelium  
T. = tartar

FIGS. 5-9.—Effect of mechanical factors in the diet on carnassial gum conditions in ferrets, as shown by the appearance of the marginal blood vessels under the slit-lamp (capillary) microscope.\*  $\times 30$ .

FIG. 5.—Normal arrangement of marginal capillaries in a ferret receiving the bread basal diet (table II) to which were added short lengths of bone plus attachments. Experimental period 8 months.

FIG. 6.—Similar region in an animal given the same basal diet but with the addition of coarse lumps of hard dog-biscuit, showing one of the earlier phases of disease. At this stage tartar deposits have just begun to impinge on the dental margin of the gum but there is little proliferation of the sub-surface epithelial papillae. Experimental period 3 months.

FIG. 7.—Marginal vessels of a ferret given the bread basal diet plus *crushed* bone. There is fairly advanced "bushing" of capillaries, indicating a stage of the disease similar to that shown histologically in fig. 4. Experimental period 3 months.

FIG. 8.—Marginal capillaries of animal fed on the bread basal diet plus finely powdered dog-biscuit. The marked engorgement and "bushing" of the capillaries indicate a phase of disease rather more advanced than that shown in fig. 7. Experimental period 3 months.

FIG. 9.—Marginal carnassial vessels of a ferret given the oatmeal basal diet (table I) for 3 months. The changes include marked vascular engorgement of the gum near the buccal groove of the adjacent tooth (between X and Y), where the impingement of heavy tartar deposits has also caused gross gingival hypertrophy.

\* Figs. 5-8 *in vivo*; fig. 9 *post mortem*.

## DENT AND GINGIVAL DISPLACEMENT IN PERIODONTITIS

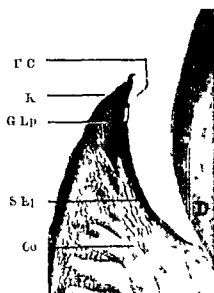


FIG 2

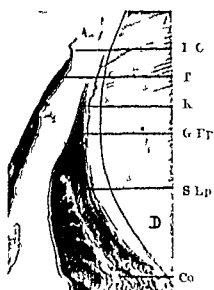


FIG 3



FIG 4

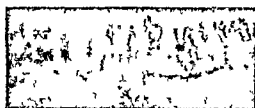


FIG 5

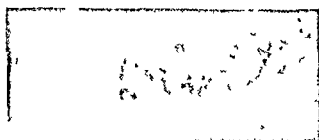


FIG 6

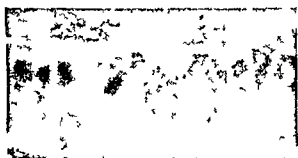


FIG 7

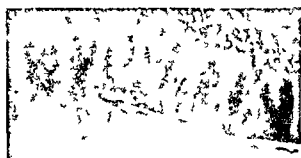


FIG 8





*Effect of diet in the cure of the disease*

This was studied in 4 ferrets (table IV). In animals 68 and 71 relatively heavy deposits of tartar and fairly severe gum disease were allowed to develop, using the oatmeal basal diet plus cod-liver oil and food yeast over a period of 11 weeks. Pieces of bone were then given

TABLE IV

*Curative effect of short lengths of bone (plus attachments) on gum disease in ferrets (oatmeal basal diet, plus cod-liver oil, plus food yeast)*

Animal no.	Bone supplements (daily)	Exptl. period (weeks)	Clinical condition of mouth at different periods in relation to addition or withdrawal of bone	
			Tartar (degree)	Gum disease (degree)
68	Nil	11	+++	++
	Rib-bone (15 g.)	5	—	—
	Nil	5	++	++
71	Nil	11	+++	++
	Rib-bone (15 g.) *	5	—	—
	Nil	7	+++	++
69	Nil	11	+++	++
	Nil	5	++++	+++
	Rib-bone (15 g.)	5	—	—
70	Nil	11	+++	++
	Nil	5	++++	+++
	Rib-bone (15 g.) †	7	—	—

\* See fig. 11.

† See fig. 10.

to each animal daily for 5 weeks. The tartar disappeared and the gums became almost normal after 7-10 days of the supplemented diet, and by the end of the fifth week no gingival abnormalities could be found clinically. The bone was then withdrawn and the ferrets reverted to the basal diet only for a further 5 and 7 weeks respectively, after which they were killed. Tartar deposits were then as heavy and gum lesions at least as severe as in the first experimental period (fig. 11, animal 71).

To the second pair of ferrets the basal diet was again given for 11 weeks, with similar results, but was continued for a further 5 weeks. Bone supplements were then given for 5 and 7 weeks respectively, after which the animals were killed. Animal 69 showed no gum disease clinically, histologically or by capillary microscopy. Ferret 70 gave similar results (fig. 10). Histologically, however, remnants of tartar could still be detected in the relatively sheltered grooves between the tooth cusps at some distance coronal to the gum.

The above findings appear to give strong support to the suggestion that the prophylactic action of bone is due to the mechanical rubbing

of the tooth surfaces against it during the gnawing away of the muscular and other attachments, which thus prevents tartar deposition.

The next stage in the investigation concerned attempts at upsetting or destroying the tartar-forming mechanism by some other method more capable of application to man.

*Effect of local surgical and chemical measures in the prevention of tartar and gum disease*

The first attempts involved frequent *scaling of the teeth* (2 ferrets). This was performed once weekly for 4 months. After 3 months tartar began to form on the treated teeth between one scaling and the next, although little or no evidence of disease could be detected in the adjacent gum with the naked eye or hand-lens. On the control (untreated) teeth of the opposite side of the mouth, tartar deposits were much heavier and gingival injection and swelling were very evident. The final effects on the gingival condition are illustrated by capillary photomicrographs of the gum taken at the end of the full experimental period (figs. 12 and 13). It is clear that, while pathological changes in the marginal capillary system are not quite so marked on the treated (fig. 12) as on the untreated (fig. 13) side, nevertheless the weekly scaling was relatively ineffective in preventing the formation of tartar in quantities sufficient to injure the gum.

The second experiment concerned treatment of the left *parotid duct with 90 per cent. chromic acid*. A few drops of acid were introduced into the opening of the duct at fortnightly intervals. Only one of the 2 ferrets survived the experimental period of 3 months, the other dying at the ninth week. In the surviving animal a considerable degree of protection was afforded to the gum adjacent to the treated duct, although even the treated side was by no means normal. It was, however, impossible to ensure that none of the acid was carried by the saliva to the adjacent tooth or gum surface. Without further tests, therefore, we are not justified in forming decisive conclusions.

*Bacteriology*

Early in the investigation, tissue changes in the ferret disease suggested the direct or indirect action of micro-organisms on the gum surface. Smears and cultures from typical lesions showed a variety of Gram-positive cocci and Gram-negative bacilli similar to those seen in human gum disease. Surface scrapings yielded abundant growth of spirochaetes and *B. fusiformis* on blood-containing media under strictly anaerobic conditions, provided the growth of Gram-positive cocci was suppressed with gentian violet. No organisms of significance were recovered from the underlying tissues and no evidence of bacterial invasion of the gingivæ was found histologically. A suspension of material from severe lesions, rich in *B. fusiformis*, spirochaetes and other organisms, on injection into the gum of normal ferrets was without pathogenic effect. When it was realised that the lesions were attributable to injury of the gum by tartar deposits, further bacteriological studies were discontinued.

## DIET AND GINGIVAL DISEASE IN FERRETS

FIG 10

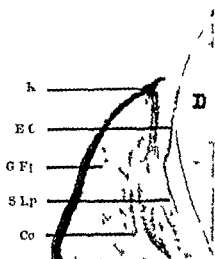
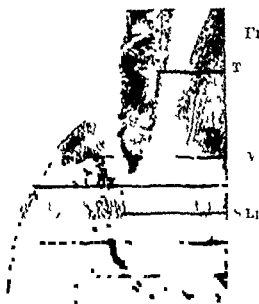


FIG 11



FIGS 10 and 11 —Photomicrographs illustrating the curative effects of short lengths of bone plus attachments on established gingival disease in ferrets  $\times 60$

FIG 10 —Appearance of the carnassial gum of animal 70 (see text) after being 'cured' by administration of bone. The tissues have almost regained their normal conditions

FIG 11 —Recurrence of tartar deposits and gingival disease in animal 71 following withdrawal of curative bone supplements

FIG 12.



FIG 13



FIGS 12 and 13 —Attempted prevention of tartar deposition and gum disease by frequent scaling of the teeth, illustrated by the condition of the gingival capillaries post mortem  $\times 30$

FIG 12 —Right upper carnassial gum region following weekly scaling of the adjacent tooth for 17 weeks

FIG 13 —Left (untreated) side of the same animal

Our thanks are due to Sir Edward Mellanby for his interest in this work, to Mr J. Smiles and Dr E. H. Schuster for help in designing the special slit-lamp microscope, to Mr Gibbs for the feeding and maintenance of the animals and for assistance in many other ways, and to the Beit Memorial Fellowship Trustees and Medical Research Council for personal and expenses grants to one of us (J. D. K.).

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# CONGENITAL MALFORMATIONS OF THE ŒSOPHAGUS

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CONGENITAL atresia of the Œsophagus with tracheal fistula—the commonest type of Œsophageal malformation—has been regarded in the past as an inoperable condition invariably fatal within a few days of birth. The increasing scope of thoracic surgery now renders the prognosis more hopeful; in fact surgical repair has been undertaken in a number of cases in America (Richter, 1913; Leven, 1936-37, 1940-41; Lanman, 1940; Carter, 1941; Haight and Towsley, 1943; Ladd, 1944). Grey Turner (1943-45) described the pioneer attempt in this country at direct suture of the Œsophageal segments after closure of the fistula. He found four examples of Œsophageal atresia in 10,543 deliveries (1935-42) at the British Postgraduate School of Medicine—a ratio of approximately 1 in every 2650 births. This incidence is sufficient to justify increasing attention to the condition.

The post-mortem records of the Royal Hospital for Sick Children, Glasgow, furnish 38 examples of Œsophageal malformation in 6916 autopsies between 16th February 1915 and 25th September 1944. These cases, which form the subject matter of this paper, are classified as follows:—

Congenital atresia with tracheal fistula . . . . .	23
Congenital atresia without tracheal fistula . . . . .	1
Stenosis . . . . .	6
Dilatation . . . . .	6
Diverticulum . . . . .	1
Muscular hypertrophy . . . . .	1

## CONGENITAL ATRESIA WITH TRACHEAL FISTULA

The lesion in all 23 cases was essentially obliteration of the middle portion of the gullet, blind ending of the upper segment and communication of the lower with the trachea in the region of the bifurcation. In one instance the fistula opened about  $\frac{3}{4}$  in. above this point. The proximal segment of the Œsophagus was generally dilated to form a small, rather thick-walled pouch approximately 1-1½ in. long; the distal part was generally of narrow calibre, especially at its origin,



and the gap, of variable length, averaged 1-2 cm. In several cases the two portions, though contiguous, were entirely separate. Five cases showed slight variation from the usual pattern in that the absent portion of the œsophagus was represented by an impermeable cord 1 cm. or more in length.

Not all patients dying in hospital come to autopsy, so the clinical records from 1915 onwards were searched for further examples of œsophageal atresia and 6 more cases with suggestive clinical findings were discovered. The series thus totals 29 cases—18 male, 11 female.

In these infants the typical history, dating from birth, is one of coughing, choking and cyanosis on attempting to swallow, since milk overflowing from the upper blind pouch enters the air passages. As a rule much frothy mucoid fluid is present in the nasopharynx. Frequently the upper part of the abdomen is tympanitic owing to the presence of air in the stomach. Nothing but meconium is passed per rectum. The presence of œsophageal atresia is readily confirmed by X-ray examination after the passage of a catheter which is held up generally about 9-12 cm. from the teeth. The practice of giving barium sulphate by mouth is contra-indicated (Lanman, Ladd) since it acts as a toxic irritant if aspirated; they regard iodised poppy-seed oil as safer. However, in Lanman's opinion simple X-ray examination after the passage of a soft rubber catheter is adequate for diagnosis.

Since infants thus affected are unable to swallow, they would die of starvation; but aspiration bronchopneumonia generally supervenes. Carter remarks that copious secretion pours from the upper pouch when it is brought to the surface for drainage.

Of the 23 infants coming to autopsy, none had healthy lungs. Aspiration pneumonia, generally extensive, was present in 18, patchy collapse in 3, hypostatic congestion in 1 and streptococcal empyema with pulmonary collapse in 1. Other developmental defects were associated with the œsophageal atresia in 4 instances. In one there was congenital atresia of part of the small bowel and the middle lobe of the right lung was missing. Another infant had severe congenital malformation of the heart and malformations of the spleen and right hand. In a third, dextrocardia was diagnosed on X-ray examination. The fourth had rectal atresia, the rectum forming a large cul-de-sac distended with meconium. There was no communication with the anal canal, which was patent though narrow. The remaining 18 cases examined *post mortem* were free from associated congenital defects and there was no mention of any external abnormality in the other 6; 24 infants died within 8 days of birth, 1 survived for 12 days and the remaining 4 for slightly shorter periods. Gastrostomy was performed in 7 cases but all died shortly after. This operation alone is useless, since, owing to the tracheal fistula, the stomach contents regurgitate upwards into the air passages and induce bronchopneumonia. In the case with a defect of the small bowel gastrostomy was combined with anastomosis between duodenum and colon.

## OTHER ABNORMALITIES OF THE ŒSOPHAGUS

The 15 remaining autopsy cases presented a variety of œsophageal malformations. In some instances the anatomical anomaly appeared slight in view of the severity of the symptoms—persistent dysphagia and vomiting, often with great emaciation. In such patients cardio-spasm probably played a part. A short description of the individual cases is given, since most of them presented features of interest. The single example of muscular hypertrophy of the œsophagus with associated hypertrophy of the pylorus and small bowel—a condition of extreme rarity—will form the subject of a separate report.

*Œsophageal atresia without tracheal fistula*

In a male infant surviving for 19 days the proximal segment of the œsophagus, comprising about two thirds of the total length, had a smooth blind lower end. The short distal portion, blind above, extended only about  $\frac{1}{2}$  in. above the diaphragm so that the gap between the segments was considerable. Death resulted from marasmus and bronchopneumonia. The infant, though able to suck at birth, regurgitated milk through the nose. Bougies were held up at the lower end of the œsophagus

*Œsophageal stenosis*

The severity of the lesion in the 6 cases forming this group is graded from virtually complete atresia to slight localised narrowing.

**Case 1**, female, 6 days, had a tight stricture with slight pouching of the gullet above it. Although the condition amounted practically to atresia, the case is placed in this group since a minute lumen existed, allowing the passage of a fine probe. Bronchopneumonia was present. The infant, who had been unable to swallow from birth, was greatly emaciated. A sister died in this hospital seven years previously at the age of 13 days. At autopsy no œsophageal lesion was found, though there was a history of inability to swallow from birth.

**Case 2**, male, 5 months, very marasmic, had marked stenosis due to an encircling band of fibrosis with pouching of the mucosa above. The infant had been healthy at birth. Regurgitation began at the age of 6 weeks on weaning, with increasing dysphagia and progressive emaciation.

**Case 3**, female, 3½ years, showed a stricture about 1 in. in length high up in the œsophagus, which was slightly dilated above it. The constricted portion of the wall was smooth and free from disease. Absence of scarring was suggestive of a congenital origin. In addition a small semicircular diaphragm was present at the pharyngo œsophageal junction. The child had been healthy prior to an attack of severe emesis at the age of 3 years. Thereafter attacks of vomiting increasing in severity recurred at ever shorter intervals and emaciation became pronounced. On admission a stomach tube could be passed only 6 in. from the teeth and X-ray showed œsophageal stenosis. Gastrostomy was performed but the child died next day.

**Case 4**, female, 5 years, showed definite œsophageal narrowing at the level of the tracheal bifurcation. Below this the wall was much thickened. There was also a "thoracic stomach". The child, a microcephalic, was much emaciated. Feeding had been difficult from birth and she had never been able

to swallow solids. The passage of bougies gave only temporary relief. Death followed a prolonged period of persistent vomiting.

**Case 5**, female, 5 months, showed considerable narrowing of the œsophagus below the tracheal bifurcation with dilatation above. Bronchopneumonia was present. There was a history of dysphagia with vomiting since birth, slight at first but recurrent and increasing in severity. Thick mucus was vomited along with the feeds.

**Case 6**, male, 4 months, had a slight stricture at the level of the tracheal bifurcation combined with localised hypertrophy affecting the last 3 cm. of the œsophagus and ceasing abruptly at the cardia. In hospital œsophageal obstruction was confirmed by a barium meal. Vomiting had been present since birth. The passage of bougies afforded relief so that the child was able to retain feeds but fatal gastro-enteritis supervened.

### *Œsophageal dilatation*

In this group of 6 cases the anatomical lesion was again relatively slight, consisting of simple dilatation, localised or diffuse, unassociated with stricture. There was slight muscular hypertrophy of the gullet wall in some instances.

**Case 1**, female, 14 weeks, a twin, showed merely dilatation at the lower end of the œsophagus. Bronchopneumonia affected both lungs. The child had dysphagia with partial regurgitation. A test feed in hospital showed ability to swallow, but the feed was returned. X-ray examination after a barium meal suggested cardiospasm.

**Case 2**, male, 16 weeks, a mongol, had a fusiform dilatation of the œsophagus with thickening of its wall. The mucous membrane, which was red and smooth, was thrown into prominent transverse ridges. Bronchopneumonia was present and the child was jaundiced and much emaciated. There was no history of vomiting or dysphagia. The œsophageal lesion was an accidental post-mortem finding.

**Case 3**, male, 10 months, showed combined hypertrophy and dilatation of the œsophagus, most marked in the middle third where the diameter was about twice the normal. At the lower end there was present a shallow oval acute ulcer which had given rise to repeated severe hæmatemesis shortly before death; the stomach and duodenum contained blood at autopsy. Early bronchopneumonia and double otitis media were present. There was a history of vomiting at least once daily since birth. Latterly emesis had been more severe and there had been great loss of weight.

**Case 4**, male, 8 months, had a funnel-shaped œsophagus broadest at the lower end. Bronchopneumonia was present. The infant thrived till the age of 5 months, when vomiting began and continued. Slight hæmatemesis occurred before death.

**Case 5**, female, 5 months, had dilatation and hypertrophy of the last inch of the œsophagus. Several small acute ulcers were present about the junction of the normal and dilated portions. There was a history of vomiting from birth with progressive emaciation. Repeated hæmatemesis occurred before death.

**Case 6**, female, 9 years, showed considerable dilatation with slight muscular hypertrophy of the whole gullet, which was of approximately constant width except for a slight constriction about  $\frac{1}{2}$  in. in length at the level of the tracheal bifurcation. The child was breast-fed and thrived till the age of 18 months, when an attack of "congestion of the lungs" was followed by severe persistent vomiting of all food and even of water. She was admitted at the age of 2 years.

and 9 months when an œsophagoscope passed readily along the whole length of the gullet, which was normal except for slight dilatation at the cardiac end. The passage of bougies improved but did not entirely cure the dysphagia. The child was not seen again for nearly six years, during which time the condition remained stationary. After a severe prolonged attack of vomiting, which began immediately after the death of her mother to whom she was much attached, she was readmitted greatly emaciated, 57 per cent underweight. A barium meal was completely held up at the level of the bifurcation. Death occurred shortly after admission.

### *Œsophageal diverticulum*

This condition was represented in a female infant aged 9 days, who showed a large thick walled diverticulum  $1\frac{1}{2}$  in long in the anterior gullet wall at the upper end, extending down behind the trachea. The œsophagus itself was patent throughout and there was no communication with the trachea. Broncho pneumonia was present. Feeding from birth had been attended with great difficulty, as the child choked and turned blue on taking food. Gastrostomy was performed but death occurred some hours later.

### DISCUSSION

Abel (1929) has classified congenital malformation of the œsophagus into seven types—total absence, doubling, œsophago tracheal fistula, partial obliteration, diverticulum, simple congenital stricture and stricture due to a membrane or valve. The present series represents all but the first two varieties, both extremely rare, and includes an example of an additional abnormality consisting of muscular hypertrophy of the œsophagus.

### *Œsophageal atresia with tracheal fistula*

According to published figures approximately 70 per cent of all congenital malformations of the œsophagus are of this type. Fischer (1926) gives the average duration of life as 2.7 days. In the exceptional case reported by Schmidgall (1915) life was prolonged for 28 days.

Surgical repair offers the one slender chance of survival to these infants, many of whom suck eagerly and are well nourished and vigorous at birth, though liable to the early onset of pneumonia as in 18 of the 23 cases coming to autopsy in the present series. Various types of operation, described in detail by the authors quoted, have been devised to surmount the great technical difficulties inherent in the problem of reconstruction of the infantile œsophagus. Another factor which must be taken into account in deciding whether to operate is the presence of associated congenital malformations. These occurred only in 4 of our 23 cases, but the recorded incidence is generally much higher (Plass, 1919, Fischer, 1926, Ladd, 1944). Atresia ani is the most frequent single concomitant defect. Multiple errors of development in the same patient are recorded in the papers

of Meusburger (1910), Harris (1922-23), Marcus (1923) and Gruenwald (1941).

The genesis of œsophago-tracheal fistula should be explicable on embryological grounds since it is a typical malformation, i.e. it is of frequent occurrence and standard pattern though not of uniform severity, since gradations exist between mild and severe forms (Gruenwald, 1940). Atypical abnormalities on the other hand may arise erratically from some such accidental cause as amniotic adhesions. Gruenwald found the usual combination of œsophageal atresia and tracheal fistula already well developed in a 9 mm. human embryo. In his opinion the essential cause of the malformation lies in the premature lengthening of the respiratory tract before separation of the œsophagus is complete, so that the rapidly growing respiratory tube draws out the corresponding portion of the œsophagus into a narrow strip of tissue incorporated into its own dorsal wall. This process results in atresia of the œsophagus and tracheo-œsophageal fistula. From the lower end of this composite tube arises the normal distal portion of œsophagus. The upper pouch, he assumes, develops from the part of the primitive tube attached to the pharynx. On the extent of œsophageal separation depends the level of the fistulous opening into the trachea, the severest type of malformation occurring when the lower portion of œsophagus communicates with the tracheal bifurcation.

As regards ætiology, Pearson (1914) and Still (1927) associate certain congenital defects and diseases with order of birth. Thus Still found that among 400 cases of congenital pyloric stenosis, 45.5 per cent. were first-born (including still-births and miscarriages) and there was a steep fall from first to second and subsequent children. Mongolism, on the other hand, occurred in high proportion in later born children. He attributes congenital deformity at the extremes of reproductive life to imperfect function and failing function respectively. Other congenital defects, e.g. cardiac and cerebral, he found occurring with undue frequency among first-born children. In a series of 160 varied malformations, including 5 congenital obliterations of the œsophagus or bowel and 5 of the bile-ducts, 41.8 per cent. were first-born, a proportion indicative in his opinion of some special liability in the first pregnancy to anomalies of development under the influence of toxæmia, to which primagravidae are particularly prone. Pearson also gives evidence, based on large series of figures, that first-born infants reaching term are physically inferior to those of later sequence.

The present series affords no evidence in support of this contention. Of the 12 cases of œsophageal atresia with tracheal fistula in which order of birth is mentioned only 1 was a first-born child. In the group of 15 subjects with varied œsophageal defects, order of birth was stated in 12, of whom only 2 were first-born children.

*Œsophageal atresia without fistula*

In this rare condition there is simple obliteration of the lumen of the Œsophagus. In a single example occurring in the present series the long proximal portion had a smooth blind end and the short distal segment formed a minute cul-de-sac closed above. The condition recalled Thomas Gibson's classical 17th century description of one of the earliest recorded cases of Œsophageal atresia (Gibson, 1697, p. 239): "The isthmus (between the Œsophageal segments) did not seem ever to have been hollow, for in the bottom of the upper and top of the lower cavity there was not the least print of any such thing but the parts were here as smooth as the bottom of an acorn cup". Particular interest attaches to this ancient report, not only because the clinical and pathological findings are described with precision, but also on historical grounds, since the author was the grandson of Oliver Cromwell and Physician-General of the Army.

*Œsophageal stenosis*

Morell Mackenzie (1884, vol. ii, p. 156) defines simple stenosis as "Abnormal narrowness of a limited portion of the Œsophagus, without any morbid change in any of its component tissues at the site of stricture". Six cases of the present series fall into this group. In the one with the greatest degree of constriction the channel, though present, was very minute, admitting only a fine probe. In a second, marked stenosis was due to an encircling fibrous band apparently of congenital origin, as regurgitation began at the age of six weeks. Reduction in calibre was relatively slight in the other 4 and there was no apparent disease of the wall. In 3 of these four cases some other Œsophageal abnormality was present. In one this took the form of localised muscular hypertrophy at the lower end of the gullet well below the stricture, in another of a semicircular valve-like fold of mucosa projecting into the lumen and forming a partial diaphragm, in the third of "thoracic stomach". This case was no. 7 of the series reported by Findlay and Brown Kelly (1930-31).

Obstruction by an annular membrane more or less complete or in the shape of a semicircular valve, though rare, is recorded by various authors (Morell Mackenzie, 1884, vol. ii, p. 219; Beatty, 1928; Abel, 1929). In Abel's patient—a 42-hour-old infant—mere pressure with the endoscope sufficed to rupture the thin membrane. Abel makes the important observation that the symptoms in all types of congenital malformation of the Œsophagus are almost identical; hence without a complete examination by all available methods cases amenable to operation may be missed. Simple atresia and stenosis are attributed by embryologists to developmental anomalies. According to Harris (1928-29) the Œsophagus and duodenum in early embryonic life act as parent tubes which give rise to daughter tubes—trachea and lungs in the former; pancreas,

liver and bile-ducts in the latter. Such parent tubes are liable to become weakened at the expense of the normally developing daughter tubes. Consequently proliferation of the mesoderm cells is defective and compensatory hyperplasia of the epithelium occurs, leading to complete occlusion of the lumen. It is to these two factors—deficient growth of mesoderm and overgrowth of epithelium—that these parent tubes owe their “pathological predestination”. The muscle coats arising in the deficient mesoderm are liable to be weakened or to be partially replaced by fibrous tissue, thus encouraging diverticulosis. Normally, after undergoing partial atrophy the solid epithelial core is canalised by the development of intra-epithelial cysts which become confluent. Atresia, stenosis or intra-epithelial cyst formation may result from interference with the normal course of evolution.

### *Œsophageal dilatation*

This condition occurred in 6 cases. Here, as in some of the patients with slight stricture, the insignificance of the morbid change was surprising in view of the severe dysphagia, vomiting and emaciation which had directed attention to the œsophagus during life. Lust (1923-24) found the same discrepancy between the autopsy and clinical findings in some of his cases. These minor anatomical lesions appeared insufficient *per se* to account for the clinical symptoms, particularly in view of the fact that a spindle-shaped dilatation of the whole gullet in one case of the present series had shown no clinical symptoms. Likewise D'Silva (1944) discovered gross œsophagectasia accidentally at post-mortem in an adult. Simple stenosis also may exist without causing dysphagia, as in the case reported by Brown Kelly (1931) of a 35-year-old man dying of cerebral hæmorrhage in whom marked stenosis at the entrance to the œsophagus had not been suspected during life. An additional factor must be sought to explain the clinical severity, and this is probably of spastic nature. According to Brown Kelly (1936) cardiospasm is not uncommon in young children. It varies in severity and duration, sometimes passing off and at others causing complete obstruction. This author describes a primary type, due apparently to congenital predisposition of which the fundamental cause is unknown. The intermittent, variable nature of the symptoms in such cases suggests transient spasm of the cardiac sphincter as a result of stimulation of the sympathetic nerves under the influence of emotional disturbance. This has a profound effect, inhibiting spasm in health but exciting it when the child is ailing or thwarted. In one of the present cases severe intractable vomiting began after the death of the child's mother. Faulkner *et al.* (1941) are convinced by their radiological experience that emotional stimulus has a marked influence on œsophageal function. Ashby (1920) and Lust (1923-24) mention that œsophageal spasm is frequently induced in susceptible infants on changing the diet, and particularly on

introducing solids which they resent. A slight anatomical defect such as simple localised stenosis or dilatation probably acts as a *locus resistantiæ minoris* where spasm tends to originate in susceptible subjects. A minute scar left after swallowing corrosive in a case of Lust's probably had the same effect.

That spasm of the œsophagus may date from birth is proved by the reports of Langmead (1919-20), Segar and Stoeffler (1930) and Negus (1936). Sudhues (1932) is of the opinion that 70 per cent of these cases originate within the first year, and half of them within the first ten days of life. This author expresses the opinion that some infantile cases of cardiospasm may be due to inhibition of vagal influence owing to injury of the vagi at birth. Examination by the usual methods, however, generally reveals no organic cause for the œsophageal spasm. It is very improbable, according to Hurst and Rake (1929-30), that this temporary disability could ever produce gross dilatation like that seen in achalasia. Here persistent closure of the sphincter occurs owing to failure of relaxation resulting from nervous imbalance, the essential cause of which Hurst regards as vagal inhibition from fibrous involvement and progressive destruction of Auerbach's nerve plexus in the wall of the œsophagus. Other workers (Cameron, 1927, Rake, 1927, Itzel, 1937) also found fibrosis of the nerve elements of Auerbach's plexus in such cases.

Two members of the group with dilatation of the œsophagus showed an acute ulcer at the lower end and gave a history of repeated severe hæmatemesis before death. In another patient slight ante mortem hæmatemesis had occurred though no ulcer was found at autopsy. Burghard (1926) also described ulceration of the œsophagus with hæmatemesis in a four months old infant with cardiospasm and spindle shaped dilatation. He comments on the extreme rarity of œsophageal ulcer in infancy.

### *Diverticulum*

Abel states that congenital diverticula with a complete muscular investment, which occur generally at the pharyngo œsophageal junction, are extremely rare in man though found in the hog and other animals. The single example in the present series had a thick walled pouch of the anterior wall of the œsophagus, the lumen of which was patent throughout and did not communicate with the trachea. Yet the symptoms present at birth were indistinguishable from those of ordinary atresia with tracheal fistula. The child died from broncho pneumonia. In cases of this type simple drainage of the pouch might save life. This type of case differs from the commoner pharyngeal pouch at the lower border of the inferior constrictor.





# THE FORMATION OF THE NUCLEUS OF STONE IN THE URINARY PASSAGES

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(PLATES XLVII AND XLVIII)

THE formation of the nucleus of a urinary stone—the origin of a concretion—has long been a subject of dispute. It is known that foreign bodies exist in all urine in the form of dead epithelium etc. and may serve as the nuclei of stones. Such nuclei are formed mainly in infected urine. Randall and his collaborators (Randall, 1936, 1937 *a* and *b*, 1940 *a* and *b*, 1941; Randall *et al.*, 1937 *a* and *b*; Randall and Melvin, 1937) consider that the formation of stone commences with the formation of a "calcium plaque" somewhere below the surface of the papilla. The epithelium overlying the plaque is dislodged, after which its surface becomes covered by stone-forming material. Hellström (1936) had previously observed the occurrence of these plaques, and Kjølhed and Lassen (1940), in post-mortem examinations, established the fact that they are very common, though they relatively seldom give rise to calculi.

Over 20 years ago the German colloid chemist Schade (quoted by Häbler, 1939) made very fine observations of concretion formation of uric acid and urates in the urine and of cholesterol in the bile. When the concentration of uric acid at a certain *pH* has become sufficiently high, freely suspended so-called micelles are formed by "tropfige Entmischung". Thus, according to Schade, such a micelle consists of a concentrated colloidal solution of uric acid, which by "ageing" crystallises out to form a small concretion. In the same way cholesterol gives rise to the formation of gall-stones.

Now both cholesterol and uric acid are substances whose capacity for forming colloidal solutions is well known. If, for example, uric acid *in vitro* is brought in contact with an equivalent amount of NaOH for the formation of acid urate in a sufficiently concentrated solution, the urate solidifies into a gel. Thus, in the first place, a colloidal sodium urate form appears, which is six times more soluble than the stable form—crystallising in needles—which originates from it (Hammarsten, 1932). Schade has predicted that other stone-forming substances also, of which the colloid forms are not known, are capable

of producing concretions in the urine by way of micelle formation, but so far there is no proof of this.

In experimental crystallisation of calcium oxalate in human urine, however, I once observed how the formation of crystals was preceded by the formation of micelles. (The micelles were also observed by Dr Tomenius, who was present in the laboratory.) Up to the present it has not been possible to reproduce this experiment.

On the other hand, the readily crystallising sulpha-compounds acetylsulphathiazole and sulphadiazine are excellent objects for the study of the formation of stone nuclei. If the urine from patients on sulpha-treatment is examined microscopically when the urine is beginning to contain a high concentration of the drug, numerous blue-shimmering drops appear in the field of vision. They most nearly

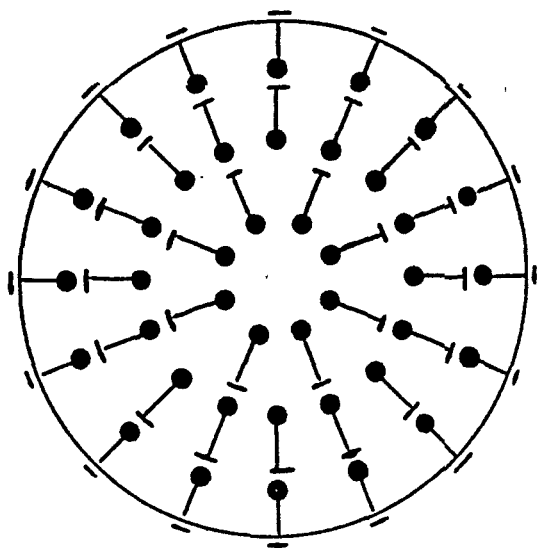


FIG. 3.—Theoretical conception of the radial structure of the micelle after crystallisation (see text): — = negative charge. ● = positive charge.

resemble drops of oil and they vary in size from a few  $\mu$  up to 50  $\mu$ . These drops are micelles (figs. 1 and 2) with high concentrations of the sulpha-drug. The micelles are extremely stable and can be centrifuged, so that it may be readily imagined that they may form cylinders in the renal channels. The surface of the micelles is charged and they are probably also hydrated, *i.e.* surrounded by a film of water, which increases their stability.

If the further development of such a micelle is followed, it is found that it sets into a crystal-like body with a radiating structure (fig. 1). When the micelle is spherical, as in the case of calcium oxalate and acetylsulphathiazole, the subsequent crystalline body also will have a spherical form (figs. 4-9). When, on the other hand, the micelle assumes the shape of a round plate, as is the case with

## URINARY CALCULUS

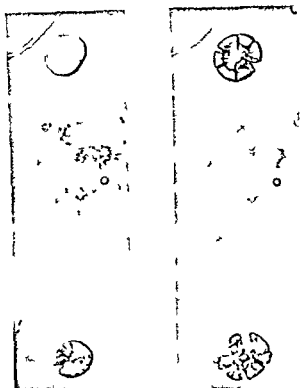


FIG 1—A Micelles of acetylsulphathiazole in human urine. The upper is about  $30\ \mu$  in diameter. Commencing crystallisation in the lower. B The same field  $5\frac{1}{2}$  hrs later. Complete crystallisation  $\times 260$

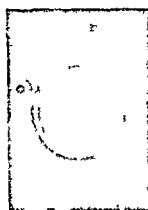


FIG 2—Sulphadiazine micelle from rat urine. Complete crystallisation  $\times 650$



FIG 4—Section of human acetylsulpha concretion. The centre consists of a former micelle, which exhibits a radiating structure. The outer crystal shows palisade formation  $\times 200$ .



FIG 5—Section of human acetylsulpha concretions  $\times 410$



## URINARY CALCULUS



FIG. 6.—Section of human acetylsulphathiazole concretions.  $\times 125$ .

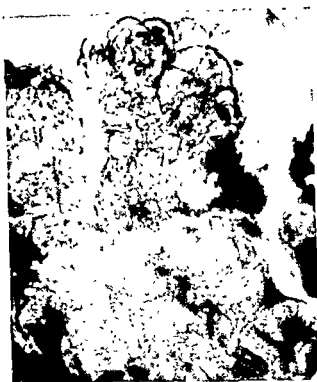


FIG. 7.—Crystalline masses of calcium oxalate from the bladder of a rat.  $\times$  = crystals resembling the most soluble of the calcium oxalate forms, i.e. the tri-hydrate. Spherical crystal forms indicating micelle structure.



FIG. 8.—Section of stratified human acetyl-sulpha concretion. The staining shows adsorption of some albumin compound in the surface layers.  $\times 635$ .



FIG. 9.—Micro-concretion of calcium oxalate in a diverticulum of bladder from a rat. Cf. fig. 8.  $\times 92$ . (Hammarsten, 1934, 1937.)



sulphadiazine, the subsequent crystalline mass will assume the same shape. It is reasonable to suppose, therefore, that already in the micelle the molecules are orientated in a way similar to that in the later crystalline form. If the disperse phase is micro-crystalline, the subsequent macro-crystalline form can perhaps be conceived as only a superstructure on the earlier formation. My speculations on the process in question have led me to the following hypothesis, of which a schematic diagram is given in fig. 3. If we imagine the configuration within a sulpha micelle formed in the urine, it is probable that the acid group of the molecule is directed towards the surface and the basic group in the opposite direction. The surface molecules are thus in a bipolar position, and the surface is conditioned by the pole facing towards the surface (fig. 3). The molecules immediately beneath the surface will be similarly orientated, "with their negative heads biting the positive tails of the surface molecule". Owing to the repulsion of similarly charged elements, the molecules will secure for themselves the greatest possible lateral room within the given space, i.e. the position of the molecules will be strictly radial. That the rate of crystallisation is very different in different planes may explain the plate form of a micelle of sulphadiazine, which substance forms flat needles when crystallising without the formation of micelles.

If sections of a sulpha-concretion are cut, a micelle will be found forming the core. Sometimes the concretion is stratified (*cf.* figs. 1 B and 8). The surface, or surface stratum, in all probability adsorbs some urinary colloid, for it stains strongly. Sometimes it is found that the radially situated crystals go right through towards the centre. Sometimes the centre preserves an almost homogeneous structure with only indications of radiation. Outside the original micelle is deposited a fresh micelle layer and so on. This is easily understood, as it is known that a colloid particle has a specific affinity for particles of the same kind as itself. As an example of such an affinity it may be mentioned that colloidal silver chloride particles are positively charged in a solution of silver nitrate by the adsorption of positively charged silver ions, but on the other hand they take a negative charge in NaCl solution owing to the adsorption of the negative Cl ions. Thus in both cases the ion which the colloid has in common with the dispersion medium is adsorbed. In the newly deposited micelle layer the molecules also lie radially. The more layers that are deposited, the higher does the concentration in them appear to be, for, after crystallisation, denser crystallisation is found the farther one moves from the centre. Finally there is produced a small stratified concretion with a surface armed with radiating crystals. Thus we find in the urine the nucleus of a stone without the presence of any foreign body, the only requisite being over-saturation with the stone-forming substance. The urinary colloids are precipitated too, and to a certain extent contribute to the solidity of the structure.

Such micelle formation is certainly of more far-reaching importance



than the mere formation of concretions. The border-line between the animate and the inanimate is not fixed, as is known from the study of certain forms of virus. It may be that in the dayspring of time the first living cell emerged as a micelle.

### Summary

The nucleus of a stone in the kidney may consist of any kind of foreign body, *inter alia* dead epithelium and bacteria. Such nuclei are formed, however, mainly in infected urine. When no infection is present, the formation of stones usually takes place in the following manner. When the stone-forming substance has reached a certain concentration, cell-like colloidal drops of the substance, so-called micelles, separate out. These are at first homogeneous and, under the microscope, resemble blue-shimmering drops of oil. Later they assume a crystalline form, with a radiating structure. Round such a micelle the concretion is subsequently built up by deposition of further layers. A number of crystallised micelles may also be held together by urinary colloids and so form the nucleus. The process has been studied on micelles and concretions of acetylsulphathiazole, sulphadiazine and calcium oxalate.

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## SHORT ARTICLES

576.8.093.31:615.778 (*Penicillin*)+615.778:25

### A SIMPLE METHOD OF TESTING THE SENSITIVITY OF WOUND BACTERIA TO PENICILLIN AND SULPHATHIAZOLE BY THE USE OF IMPREGNATED BLOTTING PAPER DISCS

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(PLATE XLIX)

Blotting paper discs impregnated with penicillin solutions have been used by various workers to detect bacterial sensitivity to penicillin and for the assay of solutions (Foster and Woodruff, 1943; Vincent and Vincent, 1943-44; Heatley, 1944; Epstein *et al.*, 1944). In such methods the discs are laid wet on the surface of an inoculated agar plate or are impregnated while lying on the agar by dropping penicillin solution on to them. Dry blotting paper discs impregnated with penicillin before drying, as described below, are more convenient to use because they dispense with the need to maintain suitable dilutions of penicillin, which must be frequently renewed on account of their instability. Because the dried discs can be stored over a long period and are always ready for use, they are likely to be most valuable in small laboratories in which a test of sensitivity to penicillin is a comparatively rare event.

#### *Method*

Discs 12 mm. in diameter are cut with a cork borer from commercial coloured blotting paper. The dye in the blotting paper has not been found to exert any inhibition on bacterial growth. Different colours can be used for different bacteriostatics.

*Preparation of desiccant.* Plaster of Paris heated to 180° C. was used at the suggestion of Dr R. I. N. Greaves, following Flosdorf and Mudd's (1938) demonstration that anhydrous calcium sulphate is a good desiccant. A little cobalt chloride, which is blue when dry but pink in the presence of moisture, is added to the plaster of Paris as an indicator.

The lid of a Petri dish is half filled with a plaster of Paris cream tinted with cobalt chloride. When the plaster has set, the lid is inverted over a Petri dish and heated in the hot air oven at 180° C. for one hour. During heating the lid should be slightly raised to facilitate drying; when the oven is turned off, the lid is lowered on to the dish by means of a glass rod.

Desiccator storage pots for the finished discs are made by half filling 2 oz. screw-capped pots with plaster of Paris-cobalt cream and then rolling them on the bench until the plaster has set in a layer on the wall. The pots are then heated at 180° C. for one hour.

Storage pots and desiccator plates are easily sealed against water vapour by running plasticine round their lids. After using a pot the seal can be quickly restored by running the finger around it. The desiccant in such a pot has remained blue after immersion for a week in the 56° C. water-bath.

*Impregnation with penicillin, and drying.* Blotting paper discs are placed in the bottom of the desiccator Petri dish so as not to overlap. The dish containing the discs is then heated to 180° C. for one hour. The discs are cooled in the refrigerator and impregnated with a cold solution of penicillin containing 50 units (manufacturer's assay) of penicillin (Pfizer) per ml. Each disc receives 0.02 ml. from a "50-dropping" pipette, that is, approximately one unit of penicillin. The lid of the Petri dish is then sealed with plasticine and the dish is placed in the -20° C. room or in the refrigerating chamber of an ordinary refrigerator, as near the coil as possible, for 48 hours. The discs are transferred by means of sterile forceps to a desiccator pot and kept preferably in the cold.

*Testing the dried discs.* An aerobic spore-bearing bacillus isolated from hay has been found to be more sensitive than the Oxford standard "H" staphylococcus and gives sharper inhibition zones. It has been used in the manner described by Foster and Woodruff (1944). A stock of spore suspension of opacity equivalent to 10,000 million *Bact. coli* per ml. is prepared from agar plate cultures and heated to kill vegetative forms. One such batch can be used over several months, 1 ml. of the spore suspension being added to 400 ml. of melted Lemco agar at 50° C. and poured into 20-25 plates, which are then stored in the refrigerator until required. A few penicillin discs from each batch of fifty are tested on these plates, two discs, 3 cm. apart, being placed on each. The plates are then incubated at 37° C. for 24 hours. Growth occurs on the surface and inhibition zones are measured from the edge of the disc to the circumference of the zone. Fig. 1 illustrates a test of this kind. A batch of discs is accepted as suitable for routine use if the sample discs give inhibition zones of 12 mm. or more.

#### *Deterioration of penicillin during manufacture and keeping of discs*

The process of drying as described leads to no appreciable deterioration of the penicillin. Ten impregnated discs were placed on spore-bearer assay plates in company with ten discs of sterile blotting paper impregnated *in situ* with 0.02 ml. of the same solution of penicillin which had been used to impregnate the dried discs and had been kept cool in the interval. Zones of inhibition were measured after 24 hours' incubation. In one such experiment the zones of inhibition around the dried discs measured on the average 13.2 mm., those around the wet discs 13.4 mm. This experiment has been repeated three times with similar results.

TABLE I

*Effect of storage at different temperatures on penicillin discs*

Temperature of storage	Time of storage								
	Days						Weeks		
	1	2	3	4	5	8	2	3	5
5° C. . . . .	...	...	...	...	...	13.8	12.7	13.1	12.2
Room temperature	12.6	11.8	11.7	...	12.0	12.8	12.9	11.6	11.4
37° C. . . . .	10.0	9.5	9.6	...	9.0	6.5	1.8	0	...
56° C. . . . .	9.4	7.6	5.4	6.0	5.8	1.8	0	...	...

The figures indicate the width of the inhibition zones in mm. Each figure is the average of eight measurements, two from each of four discs.

Since this experiment, discs kept at room temperature have been tested after three months and no deterioration has been found.

Dried impregnated discs were stored in plasticine-sealed desiccator pots for five weeks, (a) in a water-bath at 56° C., (b) in an incubator at 37° C., (c) at room temperature and (d) in the refrigerator at about 5° C.

Twenty of the discs were tested on spore-bearer assay plates before storage was started; these gave an average inhibition zone of 13 mm. At intervals, four discs were removed from each pot and tested, with the results shown in table I.

*Use of dried discs for detecting penicillin sensitivity in routine plate cultures from wounds*

A swab freshly taken from a wound is streaked on to a blood agar plate so as to produce an even distribution of colonies, as shown in fig. 2. A penicillin disc is placed on the agar surface so that its inhibitory effect can be seen both in an area in which colonies are crowded and in one in which they are sparse. After incubation, sensitive bacteria will be absent from a zone about 1 cm. wide around the disc. Typical plate cultures are illustrated in figs. 3 and 4.

*Blotting paper discs impregnated with sulphathiazole and dried*

Discs of mauve blotting paper are impregnated by a "50-dropping" pipette with 0.04 ml. of a 0.25 per cent. solution of sulphathiazole in broth. (The solubility of sulphathiazole in broth is higher than in saline, which allows such a solution to be prepared easily.) The discs are dried in the incubator at 37° C. without a desiccant and stored in sterile screw-capped pots until required. When such discs are used on blood agar cultures zones of inhibition of growth occur and presumably indicate sensitivity of the bacteria present to sulphathiazole. The absence of an obvious zone of inhibition may not imply absence of sensitivity to the drug, for blood agar contains a sulphonamide inhibitor. The use of lysed blood in the agar as recommended by Harper and Cawston (1945) apparently obviates this difficulty. Although the significance of a negative result has not been worked out, the results obtained with sulphathiazole are given because there seems no reason to doubt the significance of the inhibition zones which are commonly seen around the discs, even on blood agar cultures.

*Results with 34 swabs from 16 wounds tested with dried penicillin and sulphathiazole discs*

The swabs were cultured on 5 per cent. horse blood agar and lysed blood agar. The distribution of bacterial species, with their sensitivities to penicillin and sulphathiazole, is shown in table II. When the result for sulphathiazole differed on the two media, that for lysed blood agar was recorded.

*Summary*

1. A simple and rapid method of determining sensitivity of bacteria to penicillin on primary plate cultures by means of impregnated blotting paper discs is described.

2. The method is likely to be of special use in small laboratories in which penicillin work is only occasional, since it obviates the need to prepare and store solutions of penicillin.

3. A similar method has been tried to detect sensitivity to sulphonamides. Although the implications of a negative result have not been worked out, this method reveals a certain number of strains which are sensitive to sulphathiazole. It also is quick and convenient.

TABLE II

*Sensitivity of bacteria from 34 swabs from 16 wounds as tested with dried penicillin and sulphathiazole discs*

Bacterial species	No. of wounds	Penicillin		Sulphathiazole	
		No. of sensitive strains *	No. of resistant strains *	No. of sensitive strains *	No. of resistant strains *
Hæmolytic streptococci . . .	9	13	0	13	0
<i>Staphylococcus pyogenes</i> . . .	15	25	1	24	2
Coagulase-negative staphylococci . .	4	3	1	2	2
Coliform bacilli . . . . .	5	0	7	4	3
Diphtheroid bacilli . . . . .	11	12	1	11	2
<i>Proteus</i> . . . . .	3	0	3	3	0
<i>Pseudomonas pyocyanea</i> . . . .	2	0	3	2	1

\* The word "strain" denotes an organism derived from one swab regardless of whether or not a similar organism was isolated on another occasion from the same wound.

The work reported in this paper was done during the tenure of a grant from the Medical Research Council. I wish to thank Professor H. R. Dean and the staff of the Department of Pathology of the University of Cambridge for their help and advice, Mr R. Weeden Butler, F.R.C.S., of Addenbrooke's Hospital for his help and for giving me access to patients under his care and Mr H. P. Hudson for preparing the photographs.

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## THE ADHESIVENESS OF BLOOD PLATELETS IN RABBITS TREATED WITH DICOUMAROL

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Dicoumarol (3, 3'-methylenebis [4 hydroxycoumarin]) is now widely used in clinical medicine for preventing the formation of thrombi and for limiting the extension of any already in course of development. The drug is known to act as an antithrombotic agent by its inhibition of the production of prothrombin.

## BACTERIAL SENSITIVITY TO PENICILLIN

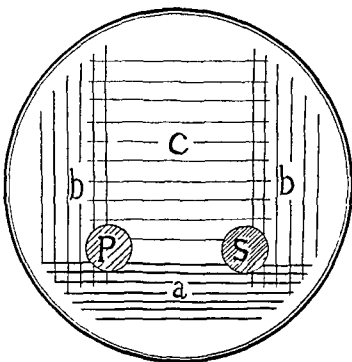
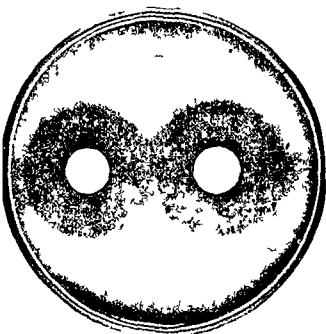


FIG. 1—Twenty four hour agar shake culture of the standard spore bearing bacillus, showing zones of inhibition of growth around two penicillin discs. Each disc had been impregnated with 1 unit of penicillin and then dried. Measurement of inhibition zones from edge of disc to circumference of zone 13 mm.

FIG. 2—Method of distributing the inoculum on a primary wound swab plate culture. The swab is rubbed over the area "a". Areas "b" and "c" are inoculated with a wire loop. Penicillin (yellow) and sulphathiazole (mauve) discs are placed at the positions "P" and "S" respectively.

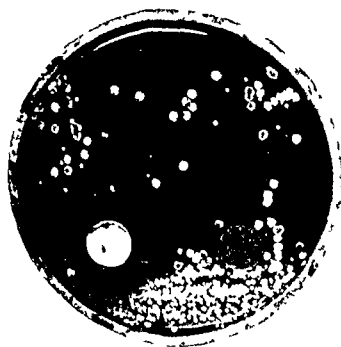


FIG. 3

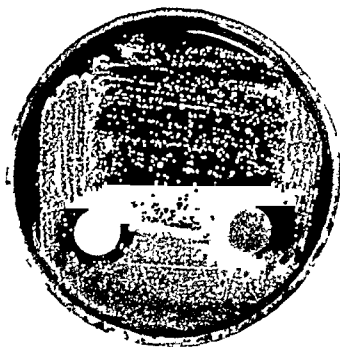


FIG. 4

FIG. 3—Case J. C. Wound. *Staphylococcus aureus* (large colonies) and diphtheroid bacillus, both sensitive to penicillin (white disc) and resistant to sulphathiazole.

FIG. 4—Case R. H. Secondly infected needle track abscess at site of administration of continuous intramuscular penicillin drip. The following colonies may be seen: (1) *Staphylococcus aureus* sensitive to penicillin, resistant to sulphathiazole. These are the largest colonies visible in the zone around the sulphathiazole disc but obscured in all other parts of the culture. (2) Diphtheroid bacillus 1', sensitive to both penicillin and sulphathiazole. This is the predominant organism in the culture. (3) Diphtheroid bacillus 2, resistant to penicillin, sensitive to sulphathiazole. A few colonies only are visible around the penicillin disc.



by the liver, but the details of its mode of operation in counteracting the formation of platelet thrombi in the circulating blood are still obscure. Earlier observations (Wright, 1941) showed that the adhesiveness of blood platelets is decreased by the addition to blood samples *in vitro* of various substances (heparin, sodium oxalate and two chlorazol dyes) which interfere with coagulation at some essential stage. The present observations upon dicoumarol, which acts as an anticoagulant only *in vivo*, were made to determine whether this drug also affects the stickiness of these blood elements.

#### Methods

The methods of counting the platelets and determining their stickiness have been described previously (Wright, 1941, 1942 and 1944). Prothrombin times were estimated by the method of Quick and Grossman (1940).

A group of 12 full grown rabbits (2.5-3 kg.), maintained upon an ample diet of fresh vegetables, bran and oats, was used for experiment. Six were injected intravenously with dicoumarol\* (0.05 mg. per kg. body weight) daily for 7 days (cf. Rose *et al.*, 1942). The other six received 1 mg. of dicoumarol per kg. by mouth daily for 7 days: this was given by stomach tube to ensure that the whole dose was administered.

Venous blood, obtained by razor cut of the marginal ear vein, was used for prothrombin times and for estimation of platelet counts and stickiness. These observations were made on the first day of administration of dicoumarol, on the 7th day, and again on the 14th day, when the drug had been withdrawn for a week.

#### Results

The average platelet counts, together with their standard errors, and the average prothrombin times are set out in the following table.

TABLE

*Effect of dicoumarol upon the platelet count (thousands per cmm.) and prothrombin time (seconds). Average of 6 observations in each group*

	Oral		Intravenous	
	Platelets	Prothrombin time	Platelets	Prothrombin time
Initial	545±28	15.5	546±30	16.0
7th day	551±18	31.9	564±27	30.5
14th day	555±22	14.5	533±23	16.5

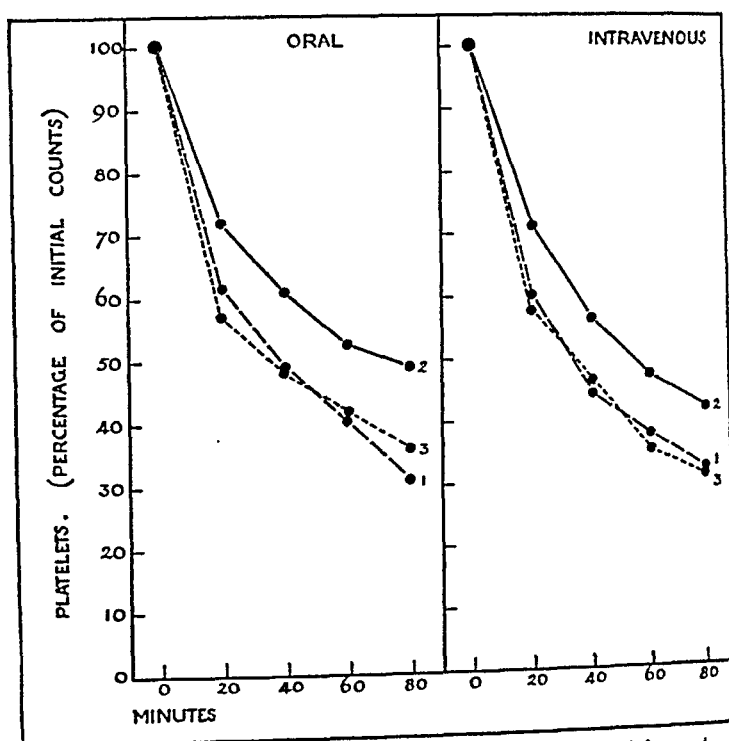
It can be seen from this table that the dicoumarol made no significant difference to the number of circulating platelets, a fact previously remarked by Wright and Prandoni (1942), Dale and Jaques (1942) and Spooner and Meyer (1944). Daily administration of the drug, however, roughly doubled the prothrombin time by the end of 7 days, though its ephemeral action can be seen from the fact that the time had reverted to normal by the 14th day.

The effect of the dicoumarol upon the stickiness of the platelets can be seen in the figure. From this it is evident that on the 7th day their stickiness had become much diminished; by the 14th day, however, when the prothrombin time had reverted to its original value, the curve showing the removal of the platelets was much the same as that found initially.

\* The dicoumarin ("Temparin") was kindly supplied by Herts Pharmaceuticals Ltd.



The changes in platelet stickiness and prothrombin time were similar in both groups of rabbits. All the animals in the intravenous group survived, but in the group which received the drug orally, two died after the 7th dose from severe gastric hæmorrhage caused by the passage of the stomach tube. The results obtained on these animals were discarded, and two other rabbits were substituted.



Number of platelets expressed as a percentage of the initial count.  
Curve 1, 1st day. Curve 2, 7th day. Curve 3, 14th day.

### Discussion

In earlier work it was found that the adhesiveness of blood platelets was lessened in the presence of various anticoagulant substances. Stickiness was inversely proportional to the concentration of anticoagulant present, and the rate of removal of the platelets from rotated blood was independent of the mode of action of the drug, whether antiprothrombin (heparin), anticalcium (sodium oxalate) or antifibrinogen (chlorazol dyes).

In the present experiments the rabbits' blood was rendered uncoagulable by creating a deficiency in prothrombin. The observations show, however, that dicoumarol, with its very different mode of action, also lessens the adhesiveness of the platelets to a degree very similar to that found with the anticoagulants previously used. The changes in stickiness and prothrombin time in the present series were, moreover, closely similar to those in 33 patients studied by the same techniques by Spooner and Meyer, to whom dicoumarol had been administered therapeutically for 6 days.

The reduction in the adhesiveness of the platelets produced by the dicoumarol again appears to be related to an interference with the mechanism of blood coagulation, since the changes in stickiness ran *pari passu* with those in prothrombin time. If the suggestion previously advanced be correct, that

the stickiness of platelets is due to a film of fibrin on their surface, the lessened stickiness in the dicoumarol-treated animals may have resulted from the interference with prothrombin formation, so that the kinase liberated in minute quantities on the surface of the platelets had no substrate on which to act. Thus interference with the coagulation process at any stage is reflected in changes in the stickiness of the platelets in the blood samples.

The action of dicoumarol in reducing platelet stickiness *in vivo* may prove to be of great importance in clinical practice. The connection between coagulation and thrombus formation is still obscure, but it is known that the platelets participate in both processes, for they form the main component of the white thrombus and are also an important source of thrombokinase. The limitation of their tendency to conglutination by the restriction of their power to promote fibrin formation on their surface might be an important factor in the control of intravenous thrombosis.

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576.8.093.31:576.852.23 (*C. diphtheriæ*)

COAGULATED PLAIN SERUM AS A DIFFERENTIAL MEDIUM  
 FOR *CORYNEBACTERIUM DIPHTHERIÆ*

G. B. FORBES and C. RICKWOOD LANE

*From a general hospital and a mobile bacteriological laboratory, B.L.A.*

Most bacteriologists use Loeffler's medium in conjunction with one of the tellurite media for throat swab culture for the detection of *C. diphtheriæ*, but a number of workers have tried, and found more effective, coagulated serum without the addition of glucose broth; one of us (C. R. L.) has obtained excellent results with such a medium for many years. Dudley (1923) was of the opinion that Loeffler's medium is distinctly inferior to plain serum.

A recent outbreak of diphtheria in this area has afforded us the opportunity of drawing a comparison between Loeffler's medium and coagulated plain serum (C.P.S.). The superiority of C.P.S. over Loeffler's medium was so striking that we are prompted to publish this short account of our findings.

*Preparation of coagulated plain serum*

Serum from horse blood obtained from the abattoir is allowed to separate, is syphoned off into sterile flasks and distributed in 2 ml. quantities into sterile screw-capped bijou bottles. The medium is coagulated in the inspissator, the temperature being raised to 75° C. and maintained at that level for 4-5 hours.

Sterilisation is effected by placing the coagulated slants in a steam steriliser and subjecting them to moist heat at 100° C. for twenty minutes on three successive days. Alternatively the serum may be Seitz-filtered in bulk under sterile conditions, tubed, and coagulated in the inspissator at 75° C. No further sterilisation is then necessary.

*Comparison of coagulated plain serum and Loeffler's serum in routine diagnostic work*

Throat swabs submitted for bacteriological examination from suspected cases of diphtheria were inoculated on Loeffler's medium, C.P.S. and Hoyle's modification of Neill's medium, using the same swab for all three. Hoyle's medium was invariably inoculated last but the order of inoculation of the serum media was reversed from time to time. After 18-24 hours' incubation at 37° C. smears from Loeffler's medium and C.P.S. were each stained by Albert's method and examined microscopically. The presence or absence of morphological *C. diphtheriae* was noted. Hoyle's medium was examined at the end of 24 hours' and again after 48 hours' incubation. Suspicious colonies on this medium were transferred to Loeffler's medium and their identity checked by film examination and by fermentation reactions. Type differentiation was carried out in all cases.

This routine was adopted until the number of swabs which yielded *C. diphtheriae* in culture on either one, two or all three media had reached a total of 100. Of these swabs 99 were positive on Hoyle's medium, 98 on C.P.S. and 74 on Loeffler. In the one instance where *C. diphtheriae* failed to grow on Hoyle's medium scanty morphological diphtheria bacilli were present on both Loeffler and C.P.S. and their identity was confirmed by subculturing the growth from these media on to Hoyle's medium. A few colonies of *C. diphtheriae* were thus isolated. The comparative results with Hoyle's medium and Loeffler are similar to those recorded by others for Loeffler and tellurite media e.g. Knox (1944).

The total number of positive cultures on C.P.S. (98) compares favourably with that on Hoyle's medium (99), the difference being in fact statistically insignificant. Moreover, tellurite-containing media, although highly selective for the growth of diphtheria bacilli fall short of the ideal in that the morphology of the organisms is frequently atypical, and C.P.S. has the advantage of giving normal morphology.

The superiority of C.P.S. over Loeffler's medium for the detection of *C. diphtheriae* in mixed culture is evident from the greater number of positive cultures obtained with it. Moreover, the total number of positives on Loeffler's medium was to a certain extent weighted by the fact that in a number of cases very scanty diphtheria bacilli were detected on this medium only after an exhaustive search of the film, prompted by the presence of numerous diphtheria bacilli in the C.P.S. film. Furthermore the advantage lay with Loeffler's medium in that this medium was inoculated before C.P.S. in the great majority of cases.

Not only does C.P.S. give a higher percentage of positive results, but in addition it usually gives a much more profuse growth of diphtheria bacilli in mixed culture. Only occasionally was the growth equally luxuriant on both media, and this occurred usually when a tag of diphtheritic membrane was adherent to the throat swab. In these circumstances a heavy growth of *C. diphtheriae* is to be expected on almost any medium. It should be noted that all films were examined by two observers working independently. Disparity in interpretation occurred seldom and then only to a minor degree. When slight disagreement did arise the films were re-examined and the result revised if necessary.

## Discussion

It should be stressed that C.P.S. possesses no advantage over Loeffler's medium for the growth of *C. diphtheriæ* in pure culture, and in fact gives a less luxuriant growth from an equal inoculation of a pure culture. In mixed culture from throat swabs, however, a growth of *C. diphtheriæ* is obtained more readily on a serum medium which does not contain glucose broth. We feel that the absence of the growth-promoting properties of glucose or broth from the medium, while depressing the growth of *C. diphtheriæ* to some extent, depresses the growth of most of the concomitant throat bacteria to a greater degree. When either glucose or broth are present the concomitant flora may overgrow or completely mask the *C. diphtheriæ*. The results of the following observations support this view.

Throat swabs from several cases of clinical diphtheria were inoculated on to the following three media: (1) C.P.S., (2) C.P.S.+0.25 per cent. glucose (equivalent to the final concentration of glucose in Loeffler's medium but with negligible dilution of the serum), (3) C.P.S.+1/3 of its volume of nutrient broth. As in the main experiment the same swab was used for all three media and the order of inoculation was varied. *C. diphtheriæ* was recovered from eight of these swabs. The proportion of *C. diphtheriæ* to other organisms was, with one exception, greater on C.P.S. than on either of the other media (table).

TABLE

Comparison between the amount of growth of *C. diphtheriæ* on coagulated plain serum with and without glucose or nutrient broth

Swab no.	Medium		
	C.P.S.	C.P.S.+0.25 per cent. glucose	C.P.S.+1/3 vol. nutrient broth
1	+	—	—
2	+++	+	—
3	++++	+	+
4	++++	+++	++++
5	+++	—	—
6	++++	+++	++
7	+++	+	+
8	++++	++	+++
Total plus value	26	11	11

++++ = profuse growth of *C. diphtheriæ* (almost pure culture).

+++ = numerous *C. diphtheriæ*

++ = number of *C. diphtheriæ*

+ = scanty *C. diphtheriæ*

— = no *C. diphtheriæ*

Throughout the main investigation horse serum was used in the preparation of both C.P.S. and Loeffler's medium. More recently we have prepared C.P.S. from ox serum and the results have been very satisfactory. Ox serum is perhaps slightly less inhibitory than horse serum to the concomitant throat flora.

Of the 100 cases referred to in the main part of the investigation 33 were of the *gravis* type, 26 *intermedius* and 41 *mitis*. C.P.S. did not appear to favour any one type more than another. Of the two cases which failed to grow on C.P.S., one was of the *gravis* type, the other *mitis*. The 26 strains which failed to grow on Loeffler's medium were composed of 12 *gravis*, 6 *intermedius* and 8 *mitis*.

The morphological characters of *C. diphtheriae* are reproduced equally well on Loeffler's medium and C.P.S. We cannot agree with Goldsworthy and Wilson (1942) when they state that the morphology of *gravis* strains on plain inspissated serum is unsatisfactory. We feel that these authors are placing too much reliance on metachromatic staining.

### Summary

1. During an epidemic of diphtheria, throat swabs were inoculated in parallel on to (1) Loeffler's medium, (2) coagulated plain serum and (3) Hoyle's medium, the same swab being used for all three inoculations.

2. Of the first 100 swabs to yield *C. diphtheriae* in culture 74 were positive on Loeffler's medium, 98 on coagulated plain serum and 99 on Hoyle's medium.

3. In mixed culture *C. diphtheriae* grows more profusely on coagulated plain serum than on Loeffler's medium. A possible explanation of this finding is discussed.

We wish to thank Colonel C. E. Eccles, O.B.E., for permission to publish this article and Sgt. F. W. C. Watts, R.A.M.C., for his technical assistance.

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### A METHOD FOR THE INHIBITION OF SWARMING OF *B. PROTEUS*

MYRA K. BEATTIE

*Indian Medical Service*

In the cultivation of micro-organisms from chronic war wounds the following method has been found useful for the prevention of swarming of *Bacillus proteus* on blood agar.

### Principle of the method

By inoculating rabbits with increasing doses of suspensions of *B. proteus* a high titre serum can be obtained. As there is a considerable degree of cross agglutination between various strains of *B. proteus* (Yacob, 1931-32), it is possible by preparing a polyvalent serum against four or five strains which show no cross agglutination, to obtain blood which will inhibit the swarming of the great majority of strains isolated from chronic wounds. However, an occasional strain may crop up in which swarming is not inhibited, and this should be incorporated in subsequent suspensions for inoculation into rabbits.

*Procedure*

Four or five strains of *B. proteus* which showed no cross agglutination were selected. Saline suspensions of these were made from agar slopes which had been subcultured from broth during the logarithmic phase and heated at 60° C. for half an-hour. The suspensions had a turbidity corresponding to Brown's no 8 or 9 standard opacity tubes. Equal parts of these suspensions were then mixed and 0.1 c.c. injected intravenously into a rabbit's ear. In our early attempts living cultures were used, starting with 0.1 c.c. and increasing the dose at 3 day intervals up to a maximum of 1 c.c., when a titre of 1:5000 to 1:10,000 was obtained. As these animals died after an interval of 3 weeks to 1 month with loss of weight and paralysis of the hind limbs it was decided to use killed cultures in the initial stages and to follow up later with living cultures of an opacity standard equal to Brown's no. 1 or 2 tube. A titre of 1:3000 was easily obtained in this way in a short time.

In order to ascertain the critical level at which swarming could be prevented, varying dilutions of serum in saline were mixed with ordinary rabbit blood agar and plates were prepared. It was found that a final dilution of 1:1000 (i.e. 1 c.c. of 1:100 dilution of serum added to 9 c.c. of blood agar) inhibited swarming, while 1:5000 was ineffective. Even after one month the titre of the rabbit's serum was sufficiently high to be used successfully without further inoculation of the animal, the titre of the serum being taken into account in assessing the amount of serum to be mixed with the blood.

A simple method was finally evolved which gave good results and which consisted in mixing one part of whole blood of an inoculated rabbit with 2 parts of ordinary rabbit blood and preparing blood agar from the mixture. Used in this way a titre of approximately 1:3000 was found to be effective. A series of rabbits can easily be maintained with sera of this titre, and the animals can be used in rotation for preparing anti swarming plates.

Cultures of hæmolytic streptococci, pneumococci, *Strep. viridans*, staphylococci, diphtheroids and *Bact. coli* were tested on these plates and no inhibition of growth was observed.

The advantages of the method are as follows. (1) *B. proteus* is not completely inhibited, only its swarming, while a stock of high titre serum is available for detecting the non spreading colonies of *B. proteus* by direct slide agglutination. (2) The method is specific for *B. proteus*. (3) It is more economical than using 8 per cent agar, which was also found effective but consumed large quantities of agar (Hayward and Miles, 1943).

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787.

## BOOK RECEIVED

### The reticulo-endothelial system in sulfonamide activity

By F. T. MAHER. Illinois Monographs on the Medical Sciences, vol. v, nos. 1 and 2. 1944. Urbana, Ill.: The University of Illinois Press. Pp. 232; 23 figs. \$3. (\$2.50 paper bound.)

This book comprises work presented as a Ph.D. thesis in 1941. Protocols are given in full and over 600 references are listed, which cover most of the relevant literature up to that date. In a historical introduction the main effects and possible mechanisms of sulphonamide activity are reviewed. This is followed by a detailed description of methods and procedures and of what the author calls "limiting experiments". These served to determine and justify the choice of experimental animal (rabbit), infecting organism (*Staphylococcus aureus*), blockading material (thorotrast) and chemotherapeutic agent (sulphathiazole). Details of dosage and the time factors to be observed are also included. Streptococci were not used because (a) no strain was found which proved uniformly virulent to rabbits, and (b) thorotrast was shown to have, *in vitro* and *in vivo*, a definitely attenuating effect on these organisms but not on staphylococci. Of particular interest among these "limiting" experiments are some dealing with the conjugation of sulphonamides. In a significant number of rabbits in which the reticulo-endothelial system was acutely blocked, acetylation of sulphanilamide was either reduced or prevented, but the same does not seem to hold true for sulphathiazole, although the figures concerning the conjugation of this drug are scantier and less convincing.

Administration of the drug alone, in therapeutic doses, to blocked rabbits produced no evidence of toxicity. Infecting inocula in doses sublethal and just lethal for normal rabbits were rapidly and uniformly fatal for acutely blockaded rabbits. Upon such rabbits sulphathiazole therapy had no protective effect in spite of the presence of blood concentrations of the drug comparable to those effective in non-blockaded infected rabbits. If sufficient time (3-4 days) is allowed to elapse after reticulo-endothelial blockage to permit proliferation and functional recovery of the R.E.S., sulphathiazole treatment again becomes effective. It is concluded that in the resistance of rabbits to staphylococcal infection the function of the R.E.S. is of primary importance, and that for sulphathiazole treatment to be effective its functional integrity is vital.

The author, then, ascribes to the R.E.S. a primary and predominant role in the mechanism of sulphonamide therapy. A theory of this mechanism is not advanced, though the author goes so far as to claim (p. 188) for the cells of the R.E.S. a chemical function in acetylating sulphonamide drugs, a claim which appears to be insufficiently substantiated. Moreover, the author wonders whether such acute R.E. blockage might not merely bring about an obstruction of the route along which the drug would normally travel to reach those cells of the host which may be the really vital ones for sulphonamide metabolism and activity, e.g. the liver cells proper.

Though the results are not always clearly defined, the experiments certainly cover an extensive field and will serve well as a guide for any embarking on similar studies.

# PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

29th and 30th June 1945

The sixty-ninth meeting of the Society was held in the Department of Physiology, the University College of South Wales and Monmouthshire, and in the Institute of Pathology, the Welsh National School of Medicine, Cardiff, on Friday and Saturday, 29th and 30th June 1945.

## Communications and demonstrations

Those marked with an asterisk are abstracted below

- M. H. SALAMAN. The occurrence of organisms of the pleuropneumonia group in the genital tracts of men and women.
- J. D. MACLENNAN and R. G. MACFARLANE. Field studies on gas gangrene.
- W. E. VAN HEYNINGEN, C. L. OAKLEY and G. H. WARRACK. The collagenase of *Cl. perfringens*.
- R. F. MENZIES and J. CRUICKSHANK. Bacterial cellulose and some possible applications.
- \*N. GOORMAGHTIGH. Observations on the juxta-glomerular complex.
- J. A. FISHER and T. F. HEWER. The relation between the adrenal cortex and hypertension.
- J. B. DUGUID. A factor in the pathogenesis of coronary atherosclerosis.
- SHEILA SHERLOCK. Aspiration liver biopsy technique and its application to diagnosis.
- A. H. CRUICKSHANK. An experimental study of the effects of the aspiration of material from the amniotic sac.
- \*E. G. BYWATERS. Hydronephrosis: histological resemblances to late changes in the crush syndrome kidney.
- L. DMOWCHOWSKY. Comparative potency of the mammary tumour agent of mice of different genetic constitution.
- A. D. T. GOVAN, A. C. FRAZER and J. J. ELKES. The aetiology of fat embolism.
- J. GOUGH, D. THOMAS and B. STILL. The effect on tuberculous cavities of occluding their draining bronchi.
- A. G. HEPPLESTON. The focal lesion of the pneumoconiosis in South Wales coal workers.
- E. J. KING. Some anomalies in the application of the solubility theory of silicosis.
- M. O. SHELTON, G. H. TOVEY and T. F. HEWER. The relationship between erythroblastosis foetalis and congenital obliteration of the bile ducts.
- S. L. BAKER. Callus luxurians in two cases of fragilitas ossium.



- \*J. BRAY. A simple method of inhibiting *Proteus* and other coliforms on routine blood agar plates.
- J. GOUGH and J. E. WENTWORTH. Focal emphysema and other lesions in Welsh coal workers.
- J. E. WENTWORTH. The hydrosulphite method of preserving museum specimens.
- T. F. HEWER. (1) Tuberculous endocarditis with tuberculous broncho-pneumonia and congenital cystic lung. (2) Accessory lobe of lung supplied by intercostal arteries.
- R. G. MACFARLANE and A. H. T. ROBB-SMITH. Some in-vitro effects of *Cl. welchii* (type A) filtrate.
- KEITH ROGERS. The employment of toluene in routine cultures.

### Abstracts

611.61—018:612.014.1

## FACTS IN FAVOUR OF AN ENDOCRINE FUNCTION OF THE RENAL ARTERIOLES

N. GOORMAGHTIGH, *Ghent*

The media of the renal arterioles is composed of fibrillar and afibrillar smooth muscle cells. The latter form the chief component of the juxta-glomerular apparatus and are granular only in those which are connected with the superficial glomeruli (young rabbits). When granular, they show evidence of glandular activity, the granules being ultimately dissolved (kidney of rabbit).

In slightly ischaemic kidneys of rabbits of which the urinary function is unimpaired, the characteristic histological changes consist in hypertrophy of the pre-existing granular cells and the appearance of granules in the afibrillar cells of the juxta-glomerular apparatus corresponding to deep-seated glomeruli. In the pre-glomerular arterioles closest to the renal capsule some of the fibrillar smooth muscle cells of the media become afibrillar and granular. A few glomeruli regress. These changes precede the rise of blood pressure and are observed from the 24th hour onwards. They are only detected after a careful examination of trichrome preparations (Bouin-Hollande or Helly fixation).

In severe renal ischaemia leading either to partial infarction or to atrophy and loss of differentiation of the renal tubules the arteriolar changes are more striking: the granular transformation of the medial cells extends towards the interlobular arterioles, the granules stain more brightly with either acid fuchsin or iron hæmatoxylin, the nuclear hypertrophy is more pronounced and there is evidence of mitotic activity.

In excessive renal ischaemia three histological features deserve special mention. (a) In the border zone of an infarct the afferent arterioles of hyalinised glomeruli remain patent for some time and almost all the smooth muscle cells of the media become granular. The intensity of this transformation underlines its relationship with the sluggish blood circulation and diminished oxygen supply. (b) In the intima of the corresponding interlobular pre-arterioles and interlobar arteries, round afibrillar cells appear, most of which contain no granules, though exceptionally a few do. This observation suggests that the function of the non-granular afibrillar cells is closely related to that of the granular cells. (c) Often the glomerular tufts are involved in the process of

granular afibrillar transformation, a fact which points to the close genetic and functional relationship of the cells of the glomerular mesangium with the smooth muscle cells of the arterioles, the glomerular tuft is an arteriolar segment adapted to the function of filtration

These arteriolar changes following stricture of the main renal artery or repeated blood tappings coincide with increased endothelial permeability and diffusion of plasma constituents in the arteriolar wall. Close to the afibrillar cells showing incipient signs of degeneration we observe the first deposits of a lipid or a siderophil substance

The granular afibrillar reaction described above declines from the third month onwards in kidneys which remain functional. In excessively ischaemic kidneys it may still be very marked about the third month but may be absent in other specimens of the same date. Two months after excessive stricture of the renal artery one is never sure in what condition the arteriolar media will be. The possibility of a succession of waves of granular change in the arteriolar wall must be contemplated

Our data concerning the transfer of these observations to human morbid histology may be briefly summarised. In normal kidneys of young subjects fixed in formol, Bouin Hollande or Helly and stained by Masson's trichrome methods, the afibrillar cells are devoid of granules. In four cases of crush wound anuria, one case of traumatic uraemia and two cases of eclamptic anuria the hypertrophy of the afibrillar cells of the juxta glomerular apparatus was considerable. Especially in cases of crush syndrome or traumatic uraemia the granules are abundant (fine fuchsinophil or coarse siderophil granules) and the cytological signs of glandular activity are evident. A striking analogy is observed with the arteriolar changes in severe renal ischaemia induced by the Goldblatt clamp. The structure of the glomerular tufts, however, is but little modified

In all cases clinically diagnosed as acute or subacute glomerulo nephritis or eclamptic uraemia, either the juxta glomerular apparatus or the glomerular mesangium is involved. The former is hypertrophic and sometimes shows signs of acute regressive change. The involvement of the mesangium, observed more often in subacute cases, is marked by the multiplication (mitotic activity) and afibrillar transformation of its cells, while its ground substance is reduced to a fine collagen network

As to the transfer of the experimental data to the morbid histology of benign hypertension, it must be kept in mind that in experimental renal ischaemia with intact urinary function the arteriolar changes are very discrete and the signs of enhanced glandular activity of the juxta glomerular apparatus transient. In benign hypertension striking cellular changes are only seen in the earliest stages of a process which extends over decades. However, the afibrillar transformation of the arterioles connected with hyalinised glomeruli resembles the corresponding changes observed in ischaemic rabbit kidneys. In other words, the progressive glomerular regression observed in human contracted kidneys entails the continual formation of transient afibrillar cells

We consider that these facts support the theory of the endocrine function of the media of the renal arterioles (Goormaghtigh, 1944). In the light of the experimental data on renal ischaemia, the histological data suggest that the granular afibrillar cells intervene in the formation of a vasopressive substance. The discovery of the glandular activity of the afibrillar smooth muscle cells supports the idea of a musculo endocrine mechanism of vasomotor activity eventually independent of the nervous mechanism

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GOORMAGHTIGH, N

1944 La fonction endocrine des artérioles rénales, *Louvain*

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## HYDRONEPHROSIS: HISTOLOGICAL RESEMBLANCES TO LATE CHANGES IN THE CRUSH SYNDROME KIDNEY

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In 11 cases of hydronephrosis produced by neoplastic blockage of the ureter, the renal tubules had ruptured and passed their colloid contents into the interstitial tissue and thence into the renal veins, where a secondary clot was sometimes formed. The extruded casts, if of any size, usually contained recognisable tubular epithelium. The appearance closely resembled that of the late stage of crushing injury or mismatched transfusion kidney. It has not yet been possible to secure clear evidence of pulmonary embolism. It is significant that this intravenous extrusion was marked only in the swollen and acutely obstructed kidney. At a later stage, large interstitial cysts (Ponfick, 1910, 1911) containing similar colloid material were seen. These were thought to be remnants of the acute stage of extrusion: they may easily be distinguished morphologically and tinctorially from the hyaline (collagenous) transformation of glomeruli characteristic of the final atrophic stage of hydronephrosis. Similar interstitial and intravenous extrusion of cast material has been seen in one case of calculous anuria and one of papillary blockage by sulphonamide crystals (see also Maisel *et al.*, 1944). Isolated cast extrusions may also be seen in nephrotic nephritis and in salyrgan nephrosis. The lesion is thought to indicate an increased gradient of pressure between the tubules, the interstitial tissue and the veins, in that order. Shearing stress may play a part in the localisation of the lesions.

Reference to such histological lesions in human pathological material has not yet been found, but the experimental production of reflux into the veins from the distended pelvis has been established in a series of papers by Hinman and his collaborators (Hinman, 1935). According to Fuchs (1931) rupture occurs at the fornix of the calyces and then into the interlobar veins. It seems probable that these experimental lesions are different from those demonstrated in human hydronephrosis, where the initial lesion appears to be tubular.

This finding throws light on the pathogenesis of the hæmoglobin and myohæmoglobin kidney. It supports the view put forward elsewhere, resulting from the experimental production of renal failure after the injection of myohæmoglobin (Bywaters and Stead, 1944-45) that "while mechanical blockage of the tubules might play a small part, . . ." myohæmoglobin ". . . acted probably in a more direct way on the tubules—perhaps by producing a physiological (resorption) blockage with a rapid rise in intra-renal pressure" (Bywaters, 1944).

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## A METHOD OF SUPPRESSING PROTEUS AND COLIFORM BACTERIA ON ROUTINE BLOOD AGAR PLATES

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While testing the effect of various volatile substances on the swarming of *Proteus* it was found that there is a significant difference between the sensitivity of some types of Gram positive organisms and those of the coliform group to the lethal action of ethyl ether. A blood agar plate inoculated with a mixture of *Proteus* and  $\beta$  hæmolytic streptococci and exposed to ether vapour for 10 minutes will, under certain conditions, yield a pure growth of streptococci. This method of obtaining pure cultures has been tested with mixed cultures as well as swabs from wounds, and without claiming too much for the method it is considered that it may, in some circumstances, be useful.

### Methods

Well dried blood agar plates were inoculated from a swab and spread evenly with a smooth loop without breaking the surface of the medium. They were then inverted over a Petri dish containing a few c.c. of ether, exposed to the vapour for varying periods of time, removed and allowed to remain tilted face downwards on the bench to allow the film of moisture which forms on the surface of the medium to dry off and finally incubated in the usual way.

All strains to be tested were grown for 24 hours in broth or peptone water and dilutions of these cultures made by adding increasing numbers of drops to a series of test tubes each containing 5 c.c. of horse serum. The swabs used for seeding the plates were dipped into the serum and the excess of fluid squeezed out on the side of the tube. As a result of preliminary trials with pure and mixed cultures a standard procedure was adopted of adding 8 drops of coliform culture and 1 drop of the culture of all other organisms to each serum tube. The optimum time of exposure to ether vapour which was lethal to the coliforms without destroying too many of the streptococci was found to be 10 minutes.

### Results

The following bacteria were tested, the number preceding the name of each organism indicating the number of strains tested, while the amount of growth following exposure to ether is shown by + (good or moderately good growth); ± (poor or very poor growth) or — (no growth).

8 *P. vulgaris*, —, 3 *Ps. pyocyanea*, —; 3 *Bact. coli*, —; 1 *Bact. typhosum*, —; 8  $\beta$  hæmolytic streptococci, +; 6  $\alpha$  hæmolytic streptococci, 3+ and 3—; 4 *Str. pneumoniae*, ±; 2 *Str. faecalis*, +; 2 *C. diphtheriae*, ±; 2 diphtheroids, +; 2 *Staph. aureus*, +; 2 *Cl. welchii*, ±.

All strains were sown from the same swabs on control plates which were not exposed to ether: these all showed a profuse growth.

*Effect on Proteus and coliforms.* Under the conditions stated the growth of *Proteus* and coliforms is usually totally suppressed. Occasionally a single colony may grow up in the thickest part of the inoculation, or a bubble hole or break in the medium may act as a nidus protecting the bacterium from the ether. If this occurs with *Proteus*, swarming will originate from the solitary colony, but only a portion of the surface is obscured and the insensitive strain can readily be isolated. The addition of increasing doses of *Proteus* culture to the serum tubes showed that the inhibitory effect of the ether is affected by the size of the inoculum. Failure may result from too heavy an inoculum.

*Effect on Gram+ cocci.* Some strains of hæmolytic streptococci appear to be more sensitive to ether than others. Of the 8 strains tested the growth was reduced from profuse on the control plate to scanty on the ether-treated plate with 3 strains, from profuse to moderate growth with 4 strains and one strain remained unaffected. Besides the reduction in number of colonies there was a slight reduction in the size of the colonies and the size of the hæmolytic zone. This was much more marked in the case of *Str. pneumoniae*, all 4 strains being ether-sensitive and showing very poor growth and abolition of the  $\alpha$  hæmolytic zone.

*Comparison with 6 per cent. agar.* A number of the swabs were tested in parallel on blood agar plates containing a high percentage of agar—6 per cent. (Lichstein and Snyder, 1941; Hayward and Miles, 1943). With an inoculum of 8 drops of the coliform culture in the serum tube, although swarming did not occur the profuse growth of the coliforms obscured much of the coccal growth and in the case of streptococci there was not a marked contrast in the numbers of isolated colonies obtainable by the two methods. In the case of *Staph. aureus* the ether method was superior in the number of colonies obtained.

### Discussion

The effect of the ether vapour appears to be that of a weak antiseptic. Following the use of this substance clinically in the treatment of wounds, peritonitis etc. by Waterhouse (1915), Topley (1915) investigated the properties of ether as an antiseptic and found that the direct immersion of *Bact. coli* in 50 per cent. ether was fatal to this organism in 3 minutes at room temperature and that the inhibition was partly determined by the size of the inoculum. In our own experiments acetone and alcohol vapour were without effect on the coliforms as were the ethers of low volatility—butyl, amyl and benzyl ether. Chloroform vapour rapidly killed both coliforms and streptococci. The principal advantage of the method is that routine blood agar plates are used and that no hæmolysis of the medium takes place. The disadvantages are that some care in the preparation and inoculation of the plates is required and that if only few streptococci are present they would probably be suppressed by the ether.

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## TWO CASES OF MIXED MALIGNANT TUMOUR OF THE BREAST

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(PLATES L-LIII)

ACCORDING to Deaver and McFarland (1918) only 21 cases of breast tumours containing both carcinomatous and sarcomatous elements had been recorded up to that date. In 1924 Fitzwilliams could find only 20 references to this condition. Since then comprehensive studies of mammary tumours have tended to omit statistics on this subject and doubt has been expressed regarding the true nature of some of the cases already recorded. The condition would therefore appear to be extremely rare. Deaver and McFarland state that giant cell sarcoma is equally rare: they could find only 28 recorded cases. I can find no more than 4 reports of cases where giant cell sarcoma has been present in combination with a carcinoma in the same breast (Schlagenhauser, 1906; Schwarz, 1913; Hedrén, 1915; Harrington and Miller, 1940).

The following cases are described not only on the count of rarity of these growths, but also because the histological diagnosis appears to be unusually precise and the similarity of case histories and subsequent development makes them of clinical interest.

### Case 1

#### *Clinical history*

A. H., aged 37, married, was admitted to the Queen Elizabeth Hospital, Birmingham, on 27.4.43, complaining of a swelling of the left breast which had been progressively enlarging for a year. For six months there had been

a discharge from the nipple. She had no pain in the breast but the nipple was tender. Previous health good.

She was a well built adult female of healthy appearance. Her general condition was good and no evidence of disease could be detected in the heart, lungs or abdominal organs. Apart from the mammary lesion the only abnormality consisted of numerous pedunculated "warts" on the face.

The left breast was greatly enlarged and pendulous and the superficial veins were distended. Dark nodules apparently cystic could be seen shining through the skin. The upper half of the breast was replaced by a well defined cystic mass. A dry crust covered the nipple and the areola had a granulating appearance. The breast was mobile and not attached to the pectoral muscles.

On 29.4.43 a local excision of the left breast was performed by Mr Sankey. The breast with the tumour was easily dissected from the chest wall and there was no suggestion of infiltration of the pectoral muscles. Convalescence was uneventful and the patient was discharged on 4.5.43. Deep ray therapy was given during June 1943 following a histological report indicating the malignancy of the condition.

She was re-admitted on 15.1.44 with a lump under the arm which had appeared three weeks previously. Examination also revealed a small mobile nodule, unattached to skin or fascia, over the second left rib in the mid-axillary line. On 20.1.44 Mr Sankey removed the pectoral muscles together with the axillary glands. She was evacuated from hospital on 25.1.44.

The patient attended regularly at the out-patient department and ray therapy clinic but in May it was found that secondary masses had appeared in both lungs and in the spine. Two months later she died at home.

### *Gross morbid anatomy*

The breast was almost completely replaced by tumour growth and was grossly enlarged, measuring  $9 \times 7$  in. in its longest axes and approximately 5 in. from the nipple to the plane of excision. The growth appeared to be well circumscribed and in parts almost encapsulated. It could be divided roughly into cystic and non-cystic parts, giving the tumour an irregularly lobulated appearance. The cysts were irregular and hæmorrhagic. They might well have been formed by necrosis of and hæmorrhage into parts of the tumour. The neoplastic tissue consisted of solid white fleshy masses bearing little resemblance to the usual carcinomatous growths of this region. In many areas it was hæmorrhagic, soft and obviously degenerate. At one point external to the nipple it had invaded the skin. There was no suggestion of irregular or widespread infiltration of the normal tissue such as one finds in carcinoma. At all points the growth was clearly demarcated from the normal breast tissue.

### *Microscopical characters*

The breast was cut into successive slices and pieces were taken for section from (a) the central portion, including the nipple, successive pieces being taken from the surface down to the plane of excision, (b) the limits of the growth and (c) the solid and cystic portions. Most of these were fixed in 10 per cent. formol-saline, others in formol-corrosive and a few in alcohol.

## MIXED MALIGNANT TUMOURS OF BREAST

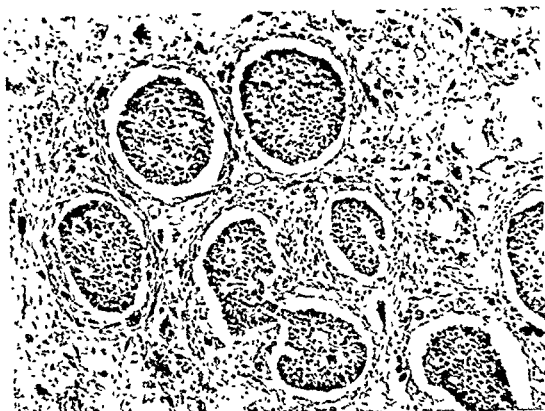


FIG. 1.—Case 1. A typical field in one of the main tumour masses, showing well defined alveoli of carcinoma cells and surrounding sarcomatous stroma. H and E.  $\times 70$ .



FIG. 2. Case 1. Part of the tumour near a "cyst". The growth is purely sarcomatous and numerous capillaries can be seen running through the mass of cells. The "cyst" is obviously the result of degeneration. H and E.  $\times 70$ .





It was at once evident on microscopic examination that two types of growth, carcinomatous and sarcomatous, were present. The carcinoma could be seen originating in the main ducts about 5 mm. below the nipple. Here the ducts were filled with masses of polygonal, obviously carcinomatous epithelial cells. They were of fairly uniform appearance and mitotic figures were not numerous. Above this level the ducts were all much dilated. The carcinoma cells could be traced down the ducts to the acinar tissue and beyond into the surrounding stroma, where they formed fairly regular, large, rounded alveoli (fig. 1). In this form they could be traced throughout most of the central portion of the tumour mass but they were absent from the tissue surrounding the hæmorrhagic cysts and from the lobulated masses at the periphery. Here and there smaller and more irregular islands of carcinoma could be seen in the central portion, but always well defined and clearly differentiated from the second type of growth.

The stroma in the superficial parts of the breast was normal, apart from slight œdema; around the ducts there was a slight lymphocytic reaction. In the deeper portion of the breast, however, the stroma had apparently undergone a sarcomatous transformation, and often the two types of growth could be seen growing side by side (fig. 1).

The sarcomatous tissue consisted of closely packed cells with little visible stroma (fig. 2). In some parts, however, it had a myxomatous appearance. The sarcoma cells presented a bewildering variety of shapes, sizes and internal structure, but several main types could be singled out for description. By far the most prominent, although not the most common, were cells of giant size, frequently multinucleated. These were of two varieties. The first was strap-shaped (fig. 3) and frequently of tremendous size, measuring up to  $300\ \mu$  in length. The cytoplasm was acidophilic and stained red with Masson's trichromic method and brown with van Gieson's stain. These cells retained the iron hæmatoxylin of Heidenhain strongly and with the use of this stain and also Mallory's phosphotungstic acid hæmatoxylin several distinct cytoplasmic structures could be made out. The most common of these was longitudinal fibrillation (fig. 3), easily demonstrated by Heidenhain's method and very prominent in preparations stained with Masson's trichrome. These fibrillæ sometimes ran the whole length of the cell and gave a feathery appearance at each end, but they were frequently confined to the margins or ran from the nuclear mass towards one end only. Very often the fibrillæ seemed to be broken up into rows of short connected rods, some of which appeared to be vacuolated. The second structure consisted of tiny dark points in the cytoplasm, frequently occurring in rows or pairs (fig. 6). The third took the form of groups of small dark bars situated at the margins of the cell and running part of the way transversely across the cell (figs. 4 and 5). More primitive examples of this consisted of small transversely placed rows of dots, again

running only part of the way across the cell. With Mallory's phosphotungstic acid hæmatoxylin a very fine pellicle could be demonstrated (fig. 4) surrounding almost every individual cell.

The nuclei also were very varied, but again certain types and characteristics could be defined. Many of the cells were multinucleated and the nuclei, both here and in the mononuclear varieties, usually occupied one of three positions. They might be central and form an irregular mass, but frequently this mass was cigar-shaped in outline; they might be found at one end of a cell, producing a rather bulbous extremity; or they might be placed along one or both margins (fig. 4). The nuclear characters varied greatly, but in the resting phase they were oval and vesicular, with a pronounced karyosome and rather coarse, darkly-staining skein of chromatin. Nuclei situated at the margins of the cell, however, which also seemed to be in a resting phase, had no such definite characters. These were usually spindle-shaped and homogeneous. Other very irregular nuclear forms were found, including some which were so intensely hyperchromatic as to suggest pre-mitotic condensation of the chromatin. In many cells mitotic figures, frequently multiple, were seen. Many of these giant cells showed branching at their extremities.

The second type of giant cell was polygonal, sometimes almost a true polygon, but more frequently roughly rounded, pear-, or (less commonly) tadpole-shaped (fig. 7). Occasionally a well defined "spider" cell was seen (fig. 8). The nuclei of these polygons showed the same variations as those described above. Longitudinal fibrillation was frequent and rows of paired dots or centrioles were common. Vacuolation was pronounced and at times of such a degree that the cytoplasm was a mere foam. Appropriate stains showed that most of these vacuoles contained glycogen (fig. 10).

The remaining cells were of relatively simple type—spindle cells of various sizes, some of them very long, and small round or polygonal cells. In some of the former faint longitudinal and cross striations were abundant. Mitoses were frequent in these cells.

Although stroma was not visible by ordinary staining methods, silver impregnation by Foot's method revealed an abundant formation of reticulum (fig. 9). This surrounded the alveoli of carcinoma cells but it had no connection with the individual epithelial cells. Examination of the sarcomatous tissue revealed an obvious contrast, as the reticulum closely invested individual cells. Unlike most sarcomata the tumour appeared at first sight to be relatively avascular, but closer investigation revealed a fine network of capillaries. The vascularity, however, was not of the degree usually found in sarcomata.

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FIG. 3—Case 1 Strap shaped cell showing branching and longitudinal striation  $125\mu$  long Heidenham's iron hematoxylin  $\times 720$



FIG. 4—Case 1 Cell showing primitive cross striation at the edges. Note that the nuclei have moved to the periphery. Part of a huge multinucleated giant cell is shown below and its fine pericellular pellicle is well demonstrated. Mallory's phosphotungstic acid hematoxylin  $\times 720$



FIG. 5—Case 1 Cell showing primitive cross striation along one edge. Mallory's phosphotungstic acid hematoxylin  $\times 720$



FIG. 6—Case 1 This shows a more common development in the giant cells. Note the rows of tiny rod and bead like structures in the cytoplasm. Heidenham's iron hematoxylin  $\times 720$



## MIXED MALIGNANT TUMOURS OF BREAST



FIG. 7.—Case 1. A tadpole cell. Stain Heidenhain's iron haematoxylin.  $\times 720$ .



FIG. 8.—Case 1. A modified spider cell showing a very complex cytoplasmic structure. Stain Heidenhain's iron haematoxylin.  $\times 720$ .



FIG. 9.—Case 1. Tumour section stained with silver. The carcinoma cells occupy the large alveoli and some of the silver has been deposited on them, but there is no sign of reticulum formation. The surrounding sarcomatous stroma shows abundant reticulum forming a fine meshwork. Foot's reticulum stain.  $\times 70$ .



FIG. 10.—Case 1. Giant cell stained by Best's carmine method for glycogen. Nucleus stained by hematoxylin and cytoplasm counterstained with light green. Some of the globules have failed to take the carmine and are stained green in consequence. The fine cytoplasmic pellicle is clearly shown.  $\times 850$ .



## Case 2

*Clinical history*

M. H., aged 47, married, was admitted to the Queen Elizabeth Hospital, Birmingham, on 1.10.43 complaining of a painful swelling of the right breast. This was first noticed six months previously as a small nodule which gradually increased in size. At this time it was painless. Two months before admission she knocked the breast severely during housework; since then it had rapidly increased in size and she had had attacks of "nagging" pain, frequently followed by a yellow discharge from the nipple. Hitherto healthy, she had had no breast trouble except after the birth of her only child. She was unable to feed it because lactation was so painful and suckling always drew blood from the breast.

She was a healthy-looking woman of good colour. Her general condition was good and no abnormality was detected in the internal organs.

The right breast was greatly and regularly enlarged and the skin over it was shiny and tense, while the superficial veins were engorged. The nipple was normal. Palpation revealed a very tense apparently cystic swelling occupying most of the breast. The consistency was denser at the base of the lower quadrants but no isolated hard mass could be felt. The swelling was slightly mobile but the mobility appeared to be restricted by the tenseness. Blood-stained fluid could be expressed from the nipple. Transillumination gave an indefinite result. No glands could be palpated and the left breast was normal.

Local mastectomy was performed by Professor Gemmill on 4.10.43. The growth was removed with the deep fascia and part of the pectoralis major. There did not appear to be any infiltration of the fascia but the growth seemed to be attached to the skin at one point. The main cyst burst at operation; it contained abundant dark brown fluid.

The patient made a satisfactory recovery and was discharged on 23.10.43. She returned to the clinic later for a course of deep X-ray therapy.

She returned once again on 9.3.44 complaining of a lump in the operation scar of three weeks' duration. Examination showed a red lump, 4 cm. in long diameter, in the lower end of the scar. On palpation the mass was found to be much larger than was at first evident and it was firmly attached to the underlying tissues. Radium needles were inserted into the growth and it was found to be cystic and contained sanious fluid.

The patient continued to attend for treatment but X-ray examination in April 1944 showed that secondary masses were developing in both lungs, and in May another secondary mass appeared involving the lower dorsal spine. The patient died on 19.6.44, and unfortunately permission for a post-mortem examination could not be obtained.

*Gross morbid anatomy*

The breast was not quite so large as the previous specimen, but the gross features were similar. A central slice showed a white solid lobulated mass of growth just below the nipple. This extended down for  $1\frac{1}{2}$  in. and was then replaced by a large hæmorrhagic cyst which had been ruptured at operation. The solid part was white and fleshy as in the previous case and throughout it there were cysts of various sizes—some hæmorrhagic, some colloid. The growth extended down to the deep fascia and at one point there was an apparent breakthrough. Generally, however, the tumour was clearly demarcated and apparently encapsulated. At one point the skin was invaded and one of the hæmorrhagic cysts lay just below the surface.



*Microscopical characters*

These were similar to those of the previous case. The tumour appeared to consist of two types of growth, one a carcinoma originating in the main ducts and spreading down into the breast tissue, the other a giant-cell sarcoma.

The carcinoma had the same well defined alveolar structure as in case 1 (fig. 11). The cells were polygonal and well differentiated and mitotic figures were not numerous. These characters were maintained throughout the growth except at one point lateral to and below the nipple, where it had become scirrhus (fig. 11) and consisted of strands of small irregular cells. They were obviously carcinoma cells and the surrounding stroma had the hyaline structure usually associated with such growths. There was no resemblance between these cells and those of the sarcomatous part of the tumour. This showed the same striking variation in cell type, but again certain main forms could be recognised in all parts. These were giant cells—strap-shaped (fig. 12) and polygonal, and smaller cells—spindles and polygons. Longitudinal striation was again a marked feature (fig. 12). Cross striation was mainly represented by slight transverse barring at the cell margins (fig. 13) and rows of centrioles. Many of the smaller spindle cells showed the crinkled extremities so commonly seen in histological preparations of muscle. The larger cells were markedly vacuolated (fig. 14) and suitable staining methods demonstrated that most of the vacuoles contained glycogen. Nuclear structure was as varied as cell form. A few cells showed a nuclear shape not seen in the previous case. This was a "spider" form with long pseudopodial processes. The nuclei in the resting phase were vesicular, with a single large karyosome, but most of the cells appeared to be in an active state.

Vascularity was not marked but there were many large thin-walled capillaries in the sarcomatous part of the growth and with these the tumour cells were in intimate contact. As in the previous case the two types of growth were intermingled and except at certain points they were only distinguishable microscopically. As before, the lateral lobules and the tissue around the cysts consisted of sarcomatous growth. Ordinary stroma was absent from the sarcomatous portion but silver impregnation revealed well marked reticulum formation.

## COMMENTARY

Gross (1887), in a review of breast sarcomata, stated that they formed 3.9 per cent. of all breast tumours. According to Cheatle and Cutler (1931) sarcomata tend to remain circumscribed and freely mobile up to a late stage, but they show a tendency to local recurrence and Gross found more than 50 per cent. of recurrences within six months of operation. Pure sarcomata of the breast have been classified into seven groups: spindle cell, round cell, mixed cell, giant cell, myxosarcoma, chondrosarcoma and osteosarcoma. Gross was of the opinion that giant cell forms were the least malignant and the

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FIG. 11.—Case 2. This shows a portion of the carcinoma just deep and lateral to the nipple. It has invaded the stroma as a scirrhus growth. The stroma has the usual hyaline characters. H. and E.  $\times 25$ .



FIG. 12.—Case 2. Half of a huge strap-shaped cell,  $300\ \mu$  long, showing longitudinal striation. Heidenham's iron haematoxylin.  $\times 720$ .



FIG. 13.—Case 2. Primitive cross-striation in a giant cell. The nuclei have moved to the margin. Mallory's phosphotungstic acid haematoxylin.  $\times 720$ .

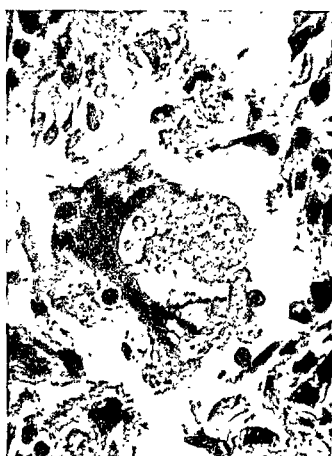


FIG. 14.—Case 2. Cell showing glycogen vacuoles. The nucleus shows pseudopodial projections which seem to extend beyond the cell boundaries. Haematoxylin and Best's carmalum.  $\times 720$ .



duration of life was longest in these cases. Riedel (1881), for instance, reported a remarkable case of giant cell sarcoma which recurred six times in twenty years and even at death there were no metastases. Geist and Wilensky (1915), on the other hand, stated that giant cell sarcomata are tumours of rapid growth, the cells showing evidence of rapid proliferation. Their survey only included two cases of this type, evidently different from those reported by Gross, and one must conclude that they were histogenetically different. Neither Gross nor Geist and Wilensky were able to determine the origin of the sarcoma cells.

In the two cases described above it must remain in doubt whether the sarcoma or the carcinoma was the cause of the metastases which were undoubtedly present. There was, however, no evidence of lymphatic permeation and the subsequent history is one of early local recurrence with later metastasis to lungs and bones via the blood stream. These facts would suggest that it was the sarcoma which had disseminated, and indeed it would seem unlikely that a well differentiated carcinoma with relatively few mitoses would produce blood-borne metastases in such a short time. If these later developments were due to the sarcomatous portion of the growth then this constituent must have been very malignant and the case falls into line with those reported by Geist and Wilensky.

As to the origin of the sarcoma cells, it seems reasonably certain that they were derived from muscle. Many of their characters are similar to those described by Cappell and Montgomery (1937). The following points have been noted, all of which agree with the statements of these authors regarding myosarcoma.

(a) The majority of the cells were long spindles or small polygons with clear vesicular nuclei and a single karyosome.

(b) Numerous giant cells, frequently multinucleated, were present. These were commonly strap-shaped, but some were bulbous, fan-shaped, tadpole-shaped or of modified spider form.

(c) The cytoplasm was acidophilic: with Masson's technique it stained red, with van Gieson's method brown. Around the cytoplasm there was a delicate sheath.

(d) Longitudinal striation was well defined in the large cells, but cross striation was only seen at their peripheries and more commonly it was represented by rows of centrioles. Very fine striation of both types was common in the smaller spindle cells.

(e) The giant cells showed marked vacuolation and most of the vacuoles contained glycogen.

(f) The giant cell nuclei were frequently irregular and showed mitotic activity. In the more differentiated cells the nuclei tended to move to the cell margins.

(g) In general the growths were not vascular and little stroma could be demonstrated by ordinary methods, but silver impregnation revealed an abundant reticulum which closely invested the individual cells of the sarcomatous portions.

## SUMMARY

Two cases of mixed malignancy in the breast are described. The carcinomatous portion of these growths had an alveolar structure and appeared to be relatively slowly growing. It was closely invested by a sarcomatous stroma the structure and staining properties of which suggest that it was derived from striped muscle.

I wish to thank Professor W. Gemmill, Professor of Surgery, Birmingham University, and Mr J. Sankey, Honorary Surgeon to Birmingham United Hospitals, for permission to publish these cases.

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# MALIGNANT TUBULAR ADENOMA IN A HORSE-SHOE KIDNEY: ITS SIGNIFICANCE WITH REGARD TO GENERAL CANCER PATHOLOGY

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(PLATE LIV)

It seems as if Cohnheim's theory of the origin of cancer has suffered something like a setback as the result of modern research work, which has been able to demonstrate the predominant part played by "irritation" in the widest sense of the term as a factor in the origin of autonomic growth, independently of the embryogenesis of the tissues concerned. This does not mean that the theory of "displaced embryonic tissues" ("versprengte Keime") either has been or can be discarded: it is, for the moment at least, simply out of focus. This becomes the more understandable, since, even in instances where dysontogenetic disturbances are still regarded as underlying the development of certain tumours (*e.g.* mixed tumours of the salivary glands and some renal blastomas in youth), it has practically never been possible to trace back their morphogenesis individually to the anticipated embryonic tissue malformation.\* On the other hand, in regions where congenital tissue irregularities are more or less frequent, the incidence of malignant tumours does not seem to be unusually high, *e.g.* in the œsophagus, with its islands of gastric mucosa, and the skin of the neck with its rests of embryonic branchial clefts, etc.†

It is this inconclusiveness in our present knowledge of the relationship between embryonic tissue disturbances and the genesis of cancer which demands further investigation and discussion. A case of malignant tubular adenoma in a horseshoe kidney which we had the opportunity of examining may be a favourable object for a study on the lines indicated. A congenital malformation is the expression of profound disturbances in the intimate structure and development of the organ or tissue involved. It remains to be considered whether

\* Teratoid tumours of testis and ovary can be excluded from consideration.

† *cf.* also, of primary sarcomas of the lymph nodes.

other pathological changes found simultaneously may not be the result of a similar and co-ordinated alteration; *i.e.* as regards our case, whether the dysontogenetic process which led to the formation of the horseshoe kidney may not also, directly or indirectly, be held responsible for the formation of the tubular adenoma. Could this be shown, it would be proof that the malignant degeneration in the adenoma had arisen on the basis of "displaced embryonic tissue".

#### CASE REPORT

We received the material (no. 893/40) as a post-mortem specimen fixed in formalin from the District Hospital, Diyarbakir (Eastern Anatolia, Turkey). The only clinical details available were that the patient was a man of 50 who had never suffered from hæmaturia. We have no information as to the post-mortem findings.

#### *Macroscopic examination*

The specimen (fig. 1) consists of a horseshoe kidney with its halves joined caudally by a wide parenchymatous bridge, showing a deep median vertebral sulcus. The right half of the organ (to the left) is the larger and contains in its centre a tumour nearly 7 cm. in diameter.

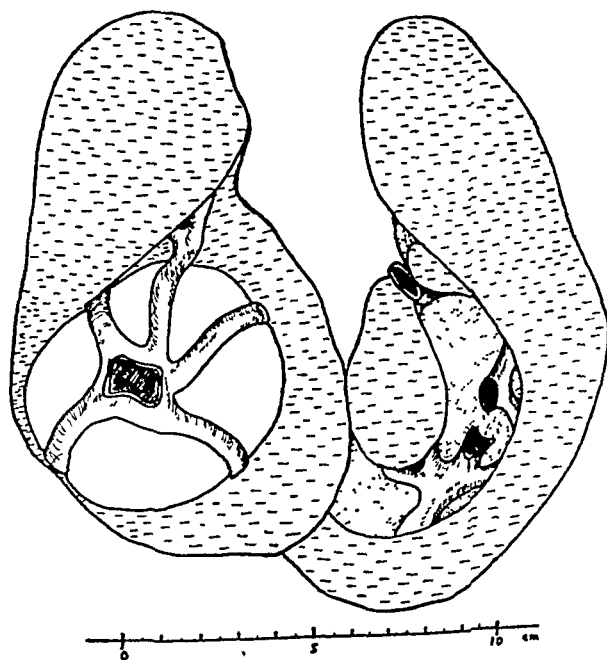


FIG. 1.—Topographic relations between tumour and horseshoe kidney (for detailed description see text).

The pelves, which are separate, cover part of the ventral surfaces. On the number and distribution of the ureters as well as of the main blood vessels no statement can be made, as they had been removed from the specimen. The elements of the left hilum lie within a crescent shaped sulcus, medially

concave and bounded laterally by a wide rampart of parenchyma, medially by a protruding lobe of parenchyma, seven calyces, distinct and separate from each other, can be followed up to their respective papillæ. The hilar region of the right half of the organ forms a big salient on top of a nearly spherical tumour. From the renal pelvis lying on its surface, five elongated calyces radiate like the spokes of a wheel and enter the organ through a slit between the tumour and the kidney tissue. Everywhere, the tumour is separated from the pelvis by a thin layer of loose connective tissue. There is no peculiarity to report about the macroscopic aspect of sections through the tumourless half of the kidney, except that it seems to be very anæmic and the cortex shows a slightly transparent yellowish tint. Various sections through the right half reveal the entire tumour, lying completely embedded in the kidney parenchyma, but separated from it by what appears to be undamaged capsule tissue. Lifting up the pelvis on the ventral side, it extends into the dorsal cortical zone and under the kidney capsule with expansive compression of all adjacent tissues. In the frontal sections the position of the extended calyces can be clearly followed around the periphery of the tumour to the points where they enclose the compressed papillæ. Nowhere are there signs of destructive invasion of the kidney, only some degree of atrophy from compression.

The tumour itself consists of rather soft, greyish white or slightly creamy homogeneous tissue, which is divided into areas of irregular size and shape by more or less conspicuous fibrous septa. There seem to be relatively few blood vessels in the stroma but some areas of hæmorrhagic infiltration as well as dry necroses are to be found.

#### *Microscopic examination*

In addition to various portions of the tumourless half of the kidney, eight blocks from the tumour itself and its neighbouring tissues were examined after paraffin embedding and routine staining.

The kidney tissue of both halves shows all the characteristic signs of advanced amyloidosis—all amyloid staining reactions being positive—with the usual nephrotic alterations. In some places tubular degeneration is rather accentuated, and here, besides hyaline casts, calcareous deposits are to be found inside the excretory ducts.

The tumour consists of a system of epithelial tubular ducts which here and there assume a rather solid aspect partly surrounded by large areas of oedematous connective tissue, loosely woven and poor in cells, partly by a few threads of collagen. There are many capillary blood vessels but only a few are of larger calibre.

Within the scheme of the general structure thus outlined, the details of the tumour present marked variation, particularly with regard to the epithelial element. Generally this takes the form of narrow, uniformly shaped tubes, more or less round on cross section and evenly distributed all over the area (fig. 2). They are lined by a single layer of mostly cubical or low columnar cells the nuclei of which are round, vesicular and of regular size. It is, however, noteworthy that even in those "quiet" regions an occasional cell contains an unusually big, irregularly shaped, hyperchromatic nucleus, or even several nuclei. Rarely there are small groups of cells, all of which present this peculiar picture. Within the lumen of some of the tubules numbers of shed nucleus containing cells are to be seen, a feature gradually becoming more obvious as the general aspect approaches greater variability of form and structure. In those places in fact the epithelial formations get closer to each other and even intercommunicate. Here and there small cystic cavities are formed containing homogeneous or cloudy masses. In some, the epithelial lining pushes out fine papillary processes into the lumen. The connective tissue becomes less conspicuous as a structural component. A change in the character of



the tumour becomes apparent when in some places the whole picture is dominated by voluminous, solidly built cell nests separated from each other by a few fibrillary threads or primitive capillaries. As already mentioned, it is sometimes hard to decide whether this epithelial structure represents solid proliferations formed "ab origine" or whether it is derived from neoplastic tubules converted into solid strands by intense proliferation of the epithelial lining, with or without desquamation. The latter interpretation is supported to some extent by the fact that here and there epithelial cells on the perimeter of the nests are disposed in unbroken pallisade formation, whereas the cells towards the centre show a completely random distribution. There can, however, be no doubt that certain formations constitute really solid tumour components. For the most part these sites present a cell picture as quiet as most of the tubular tissue. Here and there, however, quite unexpectedly, islets with pronounced anaplastic features are seen (figs. 3 and 4). This means that within certain lobule-like areas the cells become pleomorphic and contain nuclei of different sizes and staining qualities, occasionally forming most picturesque multinuclear giant cells, while mitoses are rather frequent.

Intracellular lipid is everywhere sparse and dustlike in appearance. As the material had been sent fixed in formalin, the negative result of Best's glycogen stain is without significance. There are rather important deposits of calcareous material, either as an amorphous precipitate or as concentrically layered micro-concretions, particularly near the pelvic border of the tumour.

Nowhere is there definite penetration through or infiltration of the fibrous capsule of the growth, although the borderline between the neoplasm and the medullary parts of the kidney next to the pelvis is sometimes indistinct. Parts of the tumour show extensive hæmorrhage and necrosis.

## DISCUSSION

Histologically, the tumour described is to be regarded as a tubular adenoma with certain parts bearing strong indications of malignant degeneration. Malignancy is supported by (1) the disproportion between the epithelial and stroma components as a result of one-sided over-development of the epithelial element in the areas concerned, followed by extensive loss of differentiation; (2) the pronounced anaplastic changes in the tumour cells, with increase of the nucleus-protoplasm index, the nuclei showing strong pleomorphism and exaggerated affinity for basic stains. There are also many multi-nucleated cells. These latter changes must be regarded as the expression of a "somatic mutation" of elements within the community of the "cell population" of the tumour. The mutation, i.e. the overthrow of inherited organisation, cannot mean anything but autonomy, in other words malignancy. On the other hand, the epithelial preponderance, while slightly ambiguous, might still be looked on as a criterion of rapid but coordinated proliferation.

Similar features are to be found in primary liver-cell cancer, which usually develops in the adenomas of hyper-regenerative cirrhotic livers. These adenomas undergo identical morphological changes before they acquire definite malignancy, a fact which has been confirmed experimentally through the three stages of liver cancer development in rats and mice treated with *o*-amino-azotoluene

## CARCINOMA IN A HORSESHOE KIDNEY

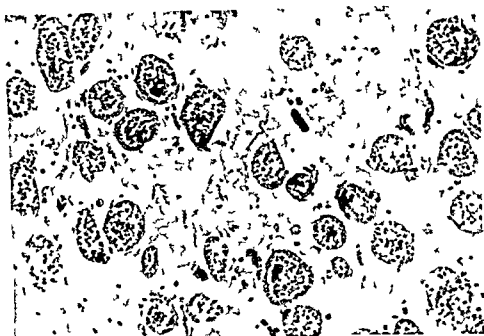


FIG 2—Benign part of the tubular adenoma. The tubular epithelial formations show a perfectly quiet cell picture with desquamated elements inside their lumina. They are separated from each other by large strands of edematous fibrillar connective tissue. Ehrlich's hematoxylin and eosin  $\times 670$ .

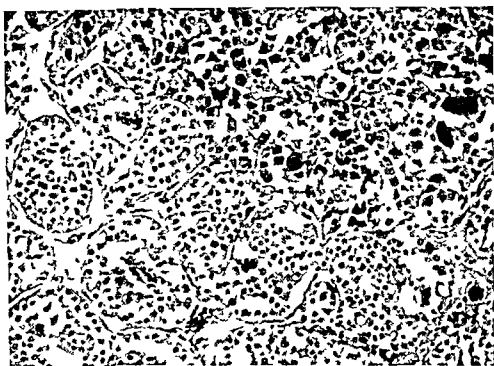


FIG 3—Malignant part of the tubular adenoma. An area showing strongly anaplastic cell features lies immediately adjacent to a benign area with closely packed tubular formations. Weigert's iron hematoxylin and eosin  $\times 670$ .

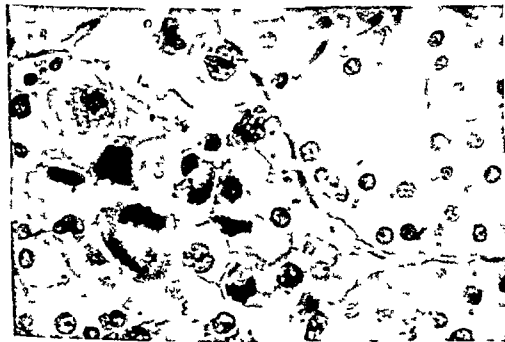


FIG 4—Details of the anaplastic cell changes including large hyperchromatic nuclei and clotted mitoses. Ehrlich's hematoxylin and eosin  $\times 670$ .



or *p*-dimethylamino-azobenzene (Edwards and White, 1941-42; Andervont *et al.*, 1942-43).

Apart from destructive infiltration of the neighbouring tissues, which in this case was neither to be ascertained nor absolutely to be denied, a decisive criterion of malignant growth is its readiness to propagate. Unfortunately, we do not know in this case whether or not metastasis had occurred, but a review of the literature (Fahr and Lubarsch, 1934) shows that in nearly all cases of similar kidney tumours so far published metastases have been described.

In relation to some of these problems, it is of outstanding importance to consider whether the formation of such tubular adenomas in the kidney might be due to hyper-regeneration and in this way analogous to the formation of adenomas in cirrhotic livers. There exist, in fact, regenerative adenomas in kidneys which have been impaired by parenchymatous alteration and prolonged processes of repair. These appear, however, to be quite different, presenting as very small, canaliculised nodules in the cortex, and often in open communication with the tubular apparatus. They generally show some at least of the peculiarities of normal kidney tubular elements. They practically never develop malignant degeneration. Tubular adenomas of the type here described, however, show no relation whatsoever to parenchymal repair and hyper-regeneration; in fact, they are generally sited amidst perfectly normal kidney tissue, with an apparent predilection for its medullary part. The reasons for the formation of these adenomas must then be sought in other more primitive disturbances in the kidney tissue.

The most widely accepted view is, indeed, to regard them as the result of an abnormality in foetal development at the time when the ureteric bud joins up with the metanephrogenic blastema and induces its differentiation and growth. In this stage, certain elements of the tissues involved may fail to establish contact and become excluded from the coordination of further development. They are left free to unfold their remaining growth potency in unusual ways.

This theory finds support in the present case from the peculiar combination of adenoma and horseshoe kidney. It is, in fact, generally agreed that horseshoe kidney is due to unusual growth impulses of the metanephrogenic tissues whereby the bilateral blastemas come into collision. In view of this, we feel that we can accept the dysontogenetic nature of the tubular adenoma. In this connection it seems to be of importance that five cases of horseshoe kidney containing tumours of unusual structure are reported in the literature (quoted by Fahr and Lubarsch), four described as sarcomas and one as a peculiar carcinoma. Although it was impossible to obtain the original publications we feel entitled to draw attention to this fact, since it indicates that the causes underlying the formation of horseshoe kidneys may occasionally involve even more profound disturbances in kidney tissue development. In our own case we

have demonstrated the existence of a "matrix" which fully corresponds with the theoretically postulated "displaced embryonic tissue" of Cohnheim as the point of departure of malignant degeneration.

There remains, however, one essential point to be discussed. The tubular adenoma with its dysontogenetic origin is only a peculiar ground on which malignant change has taken place. In the same way adenoma of the cirrhotic liver predisposes to malignancy, as do adenoma of the prostate, certain forms of adenoma of the female breast and, in rare instances, adenoma of the thyroid. The cause of the formation of these different adenomas is the potentiality for prospective growth in existent embryonic tissue, or hyper-regenerative power in impaired tissue or even unbalanced hormonal stimulation. But none of these can be regarded, by themselves, as responsible for the induction of malignancy. This is shown by the uneven ratio between the frequent occurrence of adenomas and the relatively rare incidence of their malignant transformation. Moreover, in accordance with recent research on the subject, we are justified in assuming that spontaneous cancer development is the result of an incidental and peculiar combination of various factors and influences, no one of which necessarily bears directly and specifically on the final result. This peculiar combination of factors must have created a zone of growth instability, within which, under the action of fortuitous conditions, catabiotic and anabiotic processes take place at an unusually high rate, providing a favourable milieu for frequent cell mutations (cf. Fischer, 1937). Most of these will pass unobserved, particularly under the influence of their own "lethal factor", but one or another may persist and appear in the offspring of the cell eventually affected as the germ of malignant growth.

### CONCLUSIONS

A case of malignant tubular adenoma arising within a horseshoe kidney is described and discussed with regard to its bearing on general cancer pathology. It is concluded (1) that the tubular adenoma ought to be regarded as a cancer matrix according to Cohnheim's theory of displaced embryonic tissue ("versprengter Keim"); (2) that such displaced embryonic tissues as well as hyper-regenerative, hormonal and other tissue disturbances merely provide zones of potential growth instability; (3) that in these zones "irritation"—in the widest conception of the term—may induce or release the fatal event of the cancer cell's birth as a somatic mutation; (4) that Cohnheim's theory and the "irritation" theory of cancer are not mutually exclusive, but, on the contrary, complementary.

We wish to express our gratitude to Dr İlhami Akcakoyunlu for permission to publish this case.

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associated with the giving of the milk factor by foster-nursing to low breast cancer females and oestrogen-treated males of the CBA and IFS strains (table I).

TABLE I

*The various groups of mice in which breast tissue was examined microscopically*

Strain	Sex	Nursed by	Presence of milk factor	Presence of oestrogen		Tissue examined		No. of mice examined
				Supplied by ovary	Supplied by injection	Breast	Breast cancer	
RIII	F	RIII	+	+	—	+	+	50
RIII	M	RIII	+	—	+	+	+	30
White label	F	White label	+	+	—	+	+	15
White label	M	White label	+	—	+	+	+	34
RIII	F	CBA	—	+	—	+	Very few	6
RIII	M	CBA	—	—	+	+	Very few	35
CBA	F	CBA	—	+	—	+	—	15
CBA	M	CBA	—	—	+	+	—	27
IFS	F	IFS	—	+	—	+	—	15
IFS	M	IFS	—	—	+	+	—	15
CBA	F	RIII	+	+	—	+	+	15
CBA	M	RIII	+	—	+	+	+	25
IFS	F	RIII	+	+	—	+	+	11
IFS	M	RIII	+	—	+	+	+	5

The breasts chosen for study were the right and left fourth, the inguinal gland serving as a landmark. But in removing breast tumours from these and other sites the covering and adjacent skin was included, so that it was possible in many cases to see the relation of the tumour to the rest of the breast tissue; this was found to be extremely helpful in studying the sequence of changes. Importance was attached to the necessity for obtaining the tissues before post-mortem decomposition had occurred. To this end, frequent inspections of sick mice were made so that they could be killed as late as possible in the experiment.

## THE EVOLUTION OF MOUSE MAMMARY CANCER

### *Intra-acinous carcinoma*

The acinus is the first and the most frequent site in which cancer can be observed. The cancerous change may first occur in one or two of a large group of acini (fig. 1) or in every one of a small group (fig. 2). The process usually affects small undilated acini; when acini have undergone cystic distension the epithelium becomes flat and inactive. the very reverse of the precancerous condition (fig. 3). Intra-acinous carcinoma is due to a change in the lining cells of the acini and not to a spread of malignant cells from the terminal ducts. It consists in an increase in their size and number, so that the whole acinus is swollen and filled with cells, in a change from columnar to polygonal shape and in hyperchromatism of the nuclei, many of which are in mitosis. Very early in the proliferative process the cells tend to grow in circles, indicating that their polarity has not been completely lost. Thus they become arranged in the form of small tubules within the





## PLATE LV

- FIG. 1.—CBA male, given milk factor by RIII; oestrogen-treated for 61 weeks. Intra-acinous cancer in a small number of a large group of proliferating acini. Bottom left, early lumination with formation of small tubules. Top left, solid intra-acinous carcinoma and formation of cyst filled with albuminous fluid. Connective tissue scanty.  $\times 75$ .
- FIG. 2.—RIII female breeder, aged 22 months. Intra-acinous carcinoma in all the acini of a group, showing early lumination and cyst formation. The figure shows approximately two-thirds of the whole group. Connective tissue average in amount.  $\times 120$ .
- FIG. 3.—RIII spayed female, oestrogen-treated for 64 weeks. Part of a group of proliferated acini, showing cystic distension and flat inactive epithelium, with intervening connective and adipose tissue.  $\times 150$ .
- FIG. 4.—RIII male, oestrogen-treated for 42 weeks. Hyperplastic secreting acini budding outwards from a duct in a breast where acini were scanty but duct epithelium was frequently hyperplastic.  $\times 250$ .

MAMMARY CANCER OF MOUSE

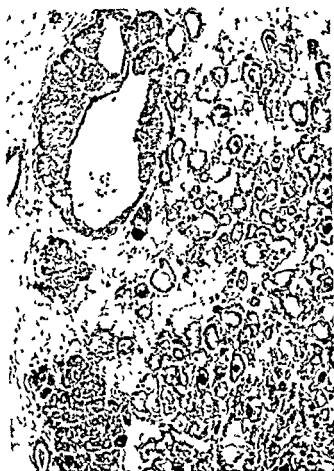


FIG 1



FIG 2

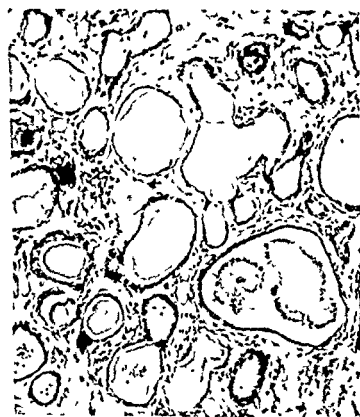


FIG 3



FIG. 4.



## MAMMARY CANCER OF MOUSE

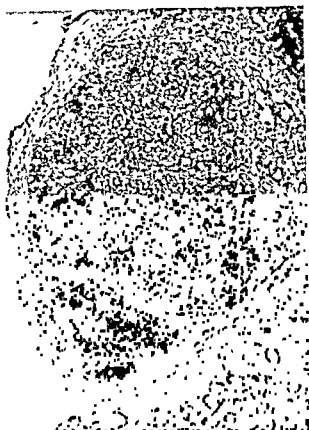


FIG. 5.—“White label” male, oestrogen-treated for 64 weeks. Small tubular adenocarcinoma composed of masses of intra-acinous carcinoma still retaining the normal acinar boundaries, lying adjacent to a large similar tumour.  $\times 65$ .



FIG. 6.—High-power view of top left acinus in fig. 5, showing large cells with nuclei varying in size and early tubule formation. Lactation in the central cells.  $\times 210$ .



FIG. 7.—“White label” male, oestrogen-treated for 47 weeks. Similar tumour to that seen in fig. 5, with (top right) commencing rupture of acinar boundaries and break-through into the connective tissue.  $\times 210$ .



FIG. 8.—“White label” male, oestrogen-treated for 96 weeks. Part of a localised area of malignant acinous proliferation forming a tumour 2 mm. in diameter.  $\times 170$ .



acinus (fig. 1), an arrangement which persists and becomes a notable feature of the fully formed tumours. This is not, however, the only mode of origin of such tubules. They appear to arise as well by outward budding, either from hyperplastic (fig. 4) or from actually cancerous acini.

The extent of the cancerous process varies from microscopic cancer in one breast to palpable tumours in as many as seven out of the ten breasts. Although in favourable sections the start may be demonstrated in one or two acini, the disease has usually a regional distribution; it is not unusual for one focus in a region to grow more rapidly than and at the expense of the others. Occasionally the whole of the breast tissue appears to be affected.

The amount of connective tissue investing the acini is very variable (figs. 1-3), but that seen in fig. 2 is average. Intra-acinous carcinoma arises more commonly in acini invested with a minimum of connective tissue than in those surrounded by a thick hyaline layer, but it has been observed in the latter condition. When the acinus swells, due to the occurrence of intra-acinous carcinoma, the connective tissue may stretch and continue to invest a lobule of the growing tumour until it has reached a considerable size (figs. 5 and 6). Thus the final tumour may be seen to be composed of a number of lobules each originally developed from a single acinus (fig. 5), but confluence of the epithelial collections within the acini may also occur and, together with new adjacent areas of intra-acinous carcinoma, may form the tumour. Owing to the power of stretching of the acinar wall, break through of the carcinoma cells into the surrounding connective tissue may not occur until a late stage in the disease (fig. 7).

In addition to the small tubular adenocarcinomas formed as described above, there is the papillary cystic type of tumour and the tumour consisting of a mixture of types. The cystic process may start quite early and affect a single acinus or a small group of acini (fig. 1), the cysts containing albuminous fluid or blood. At this stage the cancerous process may be seen to affect part or all of the wall of the acinus, the final complicated pattern being merely due to a combination of intra-acinous cancer and cyst formation.

*Malignant acinous proliferation.* Not infrequently groups of acini are seen of which the epithelium, though perfectly orderly in arrangement, is in a state of great activity. Large columnar or cubical cells with hyperchromatic nuclei, frequently in mitosis, line a slightly dilated lumen; the acini are closely packed and there is a minimum of intervening connective tissue (fig. 8). These appearances are in themselves highly suggestive of malignant change and similar groups of acini can sometimes be seen adjacent to or incorporated in a mammary cancer (fig. 9). It has not been possible to find a tumour formed entirely in this way, areas derived from intra-acinous carcinoma always being seen as well. But it is significant that this type of acinar proliferation is confined to those mice which have

received the milk factor (see p. 417) and are therefore liable to develop mammary cancer. No evidence has been obtained as to whether this type of proliferation can undergo regression. This process is quite different from that seen in fig. 10, where cystic acini of all sizes are closely packed, are quite simple in type and form a cystic adenoma.

### *Intraduct carcinoma*

This has been observed but rarely in comparison with the frequency of intra-acinous carcinoma. Small simple papillary epithelial projections into the duct lumen have frequently been seen (Burrows, 1935-36; Bonser, 1936), but I have found no evidence that these are in any way connected with the subsequent development of cancer. Papillary infoldings of epithelium (fig. 11) are also not uncommon but these seem to be due rather to tortuosity of the duct wall than to a process of new growth. Intraduct squamous metaplasia occurs on rare occasions in both females and oestrogen-treated males. This change may affect adjacent acini also. Although it has not been possible to trace the sequence of events with certainty, it seems not unlikely that it is antecedent to the formation of those fully developed cancers, some areas of which show squamous metaplasia.

True intraduct papillomata such as are common in the human subject, consisting of a connective tissue core and covering epithelium, have not been seen, but in fig. 12 a papilloma-like mass of epithelium is seen detached from the wall of the duct. Already there is lumen formation and irregularity of nuclear size and staining reaction. These formations were multiple and some had undergone frankly malignant change. In addition, in neighbouring ducts, were areas of luminated intraduct cancer and squamous cancer. There was intra-acinous carcinoma in the adjacent acini (fig. 13).

Intraduct carcinoma, arising apart from papillomata, has also been seen in areas where the surrounding or adjacent acini are affected. It cannot always be decided with certainty whether a particular structure is a duct or an acinus, as acini the seat of carcinoma often undergo early cystic distension. For example, in fig. 1 the cystic structure on the left might be either duct or acinus, but was thought to be the latter because ducts take some time to attain this size and usually contain inspissated secretion, whereas the secretion here is very recent. Anatomically, also, it seemed to be one of a group of acini.

### EFFECT OF THE MILK FACTOR

When cancer-resistant CBA mice were foster-nursed by RIII females, a high incidence of breast cancer was observed in the females and in oestrogen-treated males (Bonser, 1944). By comparing the breast tissue of 27 normal and 25 foster-nursed oestrogen-treated males, the specific effect of the addition of the milk factor could be observed.

## MAMMARY CANCER OF MOUSE

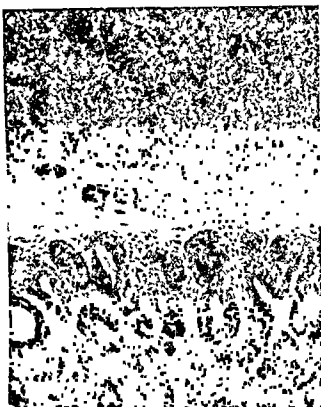


FIG 9—CBA male, given milk factor by RIII, oestrogen treated for 48 weeks. Central portion of a lobulated breast tumour with intervening connective tissue septum. Above, small tubular adenocarcinoma, below, proliferating acini forming part of the tumour and surrounded by young connective tissue  $\times 100$ .



FIG 11—CBA male, given milk factor by RIII, oestrogen treated for 48 weeks (same mouse as fig 9). Tortuosity of duct wall simulating papilloma formation  $\times 85$ .

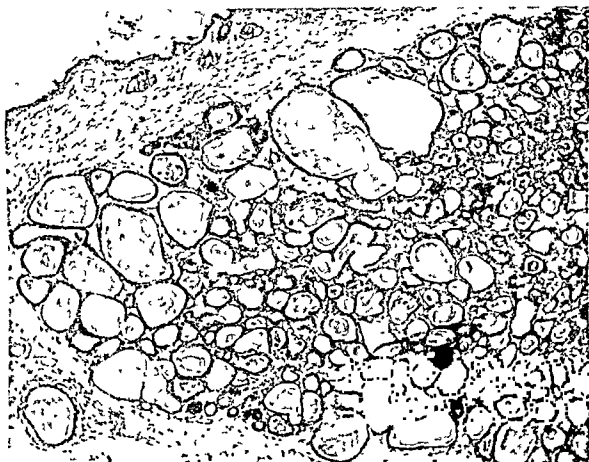


FIG 10—RIII male, oestrogen treated for 65 weeks. About one third of a cystic adenoma, showing flat inactive epithelium lining the acini  $\times 65$ .





The differences between the two groups were as follows. (1) Simple acinous proliferation or hyperplasia was of greater degree and intensity in the foster-nursed mice. (2) Intra-acinous carcinoma was seen in 19, *i.e.* in all but one of the foster-nursed mice which developed palpable cancer. It was not seen in the remaining 6 mice which did not develop palpable cancer nor in any of the normal mice. Malignant acinous proliferation (as described on p. 415) was seen in 7 mice of the foster-nursed group but in none of the normal mice. (3) Infiltrating or extra-acinous cancer occurred only in the foster-nursed mice (table II).

TABLE II

*Comparison of breast changes in normal and foster-nursed oestrogen-treated male mice*

Strain	Nursed by	Presence of milk factor	Total no of mice	Breast cancer	Intra-acinous carcinoma	Malignant acinous proliferation	Degree of acinous proliferation
CBA	CBA	—	27	0	0	0	+
CBA	RIII	+	25	20	19	7	++
IFS	IFS	—	15	0	0	0	++
IFS	RIII	+	5	2	2	0	++
RIII	RIII	+	30	21	18	4	++
RIII	CBA	—*	35	2	2	2	+

\* These mice may have received a small dose of milk factor from their own mothers.

No difference could be detected in the degree of duct distension in the two groups. Squamous metaplasia of duct epithelium occurred occasionally in both. Simple papillary infolding of duct epithelium was more commonly observed in the foster-nursed group.

Similar, though smaller, groups of IFS males and females were also examined (table II). The breast tissue of this strain differs from that of others tested in being peculiarly susceptible to the action of chemical carcinogens (Bonser, 1940; Orr, 1943). No clear-cut difference was observed in the degree of simple acinous proliferation in 15 oestrogen-treated normal males compared with 5 similarly treated males foster-nursed by RIII. In both groups it was very marked but did not attain to the degree described as malignant acinous proliferation (fig. 14). Neither intra- nor extra-acinous carcinoma occurred in the normal males, whereas 2 foster-nursed mice developed palpable tumours, in relation to which were areas of intra-acinous carcinoma (table II). Five out of 7 foster-nursed females developed mammary cancer and, in each of 3 examined histologically, areas of intra-acinous carcinoma were seen adjacent to the fully developed tumours. In 2, squamous metaplasia of duct epithelium was seen and in one, malignant acinous proliferation.

The converse of the above condition, *i.e.* the effect of withdrawal of milk factor from RIII mice by means of foster-nursing by CBA

mice, was also investigated. Groups of 30 normal and 35 foster-nursed oestrogen-treated RIII males were available (table II). It was not expected that the results would be so clear-cut, as frequently the new-born young are not removed from their mothers until some hours have elapsed, thus allowing them to receive small quantities of the milk factor. Simple acinous proliferation was of greater degree and intensity in the normal group, which received the milk factor, but the difference was less marked than in CBA mice. Malignant acinous proliferation was seen in 4 normal and 2 foster-nursed mice, the latter being the only ones in the group to develop infiltrating mammary cancer. Intra-acinous carcinoma was observed in 18 of the 21 normal RIII mice which developed palpable breast cancer and in 2 foster-nursed mice, one of which had a breast cancer. No differences were observed in the degree of duct distension or papillary infolding in the two groups, although individual variations were very great in both groups. Squamous metaplasia was not seen in this strain.

These observations warrant the conclusion that the effect of the milk factor is mainly upon the acini. The most usual sequence of events in the full development of mouse mammary cancer is:—(a) acinous hyperplasia, (b) intra-acinous carcinoma (accompanied in some cases by intraduct carcinoma) and (c) extra-acinous or infiltrating carcinoma. In two strains, RIII and CBA, the fullest degree of acinous hyperplasia was only seen when the milk factor was present; in IFS mice, acinous hyperplasia attained a very high degree even in the absence of the milk factor. Malignant acinous proliferation, though not of frequent occurrence, was seen (with the two exceptions described above) only in mice which had received the milk factor. Acinous hyperplasia seems to be a necessary preliminary condition for the initiation of cancer, as was also shown by Loeb and Suntzeff (1941b) in a very large series of mice. In general the higher degrees of hyperplasia lead more frequently to cancer, but this is not always the case. In fig. 10, for example, the formation of new acini is extreme and yet there is no suggestion that they are becoming cancerous; in fig. 2, the converse is the case. In normal IFS mice there is well marked acinous hyperplasia but in the absence of milk factor no cancerous change occurs.

The transition from hyperplasia to intra-acinous carcinoma is a gradual one. It is not possible to decide the moment of transition or whether a particular group of cells is actually malignant or not. Once intra-acinous carcinoma is established, it is not possible to estimate how long the malignant cells will remain confined within the boundary of the acinus.

## MAMMARY CANCER OF MOUSE



FIG 12—RIII male, deprived of milk factor, oestrogen treated for 58 weeks. Intraduct papilloma like mass of epithelium showing early lumen formation and some irregularity in size of nuclei. These structures were multiple and some were frankly malignant.  $\times 165$



FIG 13—Same mouse as fig 12. Above, intraduct squamous cancer, below, intraduct acinous carcinoma. On right, intraduct cancer with early lumination.  $\times 85$

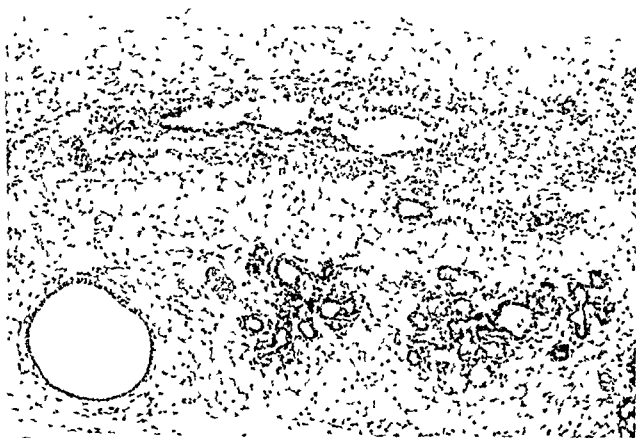


FIG 14—IFS male, oestrogen treated for 68 weeks. Above, duct showing simple papillary infolding of epithelium, below, hyperplastic acini (a process very much more active in the next field not seen here).  $\times 100$



COMPARISON OF THE STRUCTURAL CHANGES IN  
HUMAN AND MOUSE BREASTS

Muir (1941) has described the site of origin of cancer in the human breast as being either (a) within the ducts (whether as a sequel to papilloma or apart from this condition) or (b) within the acini, the former site being the more frequent. "Intra-acinous carcinoma is often merely the result of the spread of cancer cells from terminal ducts in which the malignant process has started". In the mouse the usual site of origin is within the acini, especially within those groups which are actively proliferating. Intraduct cancer occurs infrequently in the mouse, is usually limited to a localised area of one duct and is coincident with intra-acinous carcinoma in the surrounding groups of acini. No clear evidence of spread from duct to acinus or vice versa has been seen. True intraduct papillomata do not appear to occur in the mouse.

How fundamental is this difference in the site of origin in mouse and man? Intra-acinous carcinoma does occur in the human subject, but as the primary site of origin it is much less common than intraduct carcinoma. Dawson (1933) prefers to call the structures comprising the human resting lobule ductules rather than acini, a term reserved for the milk-secreting structures of lactation. Thus, in her view, the origin of all human breast cancer is in the duct system, a view not supported by most workers. In the mouse, there is no well defined lobule and the structures here referred to as acini are the structures which secrete the milk. In favourable circumstances an almost universal transformation of lactating to cancerous acini may be seen (fig. 15). As it seems clear that in the mouse the milk factor acts upon the acini, the question is raised as to whether a milk factor exists in the human subject and, if it does, whether it is of a different kind, acting upon the ducts.

In other respects there is close resemblance between the evolution of cancer in the two species. The proportion of cases in which it can be observed is greater in the mouse than in the human subject, because in the former (1) the tumours remain circumscribed until a late stage in the disease, (2) there may be several tumours in one mouse at various stages of development and (3) affected and non-affected breasts in the same mouse can be examined. In both species the origin is frequently multicentric. In the mouse this applies to different areas in the same breast and to different breasts in the same mouse. In both, the process is often a gradual one and may be seen at different stages in the same breast or in different breasts in the same animal. As described by Muir, the malignant process represents a gradually accelerating hyperplastic growth attended by de-differentiation; it is not possible to state definitely at what stage cells acquire the malignant character but undoubtedly before the break-through into the tissue spaces.

Nevertheless in the mouse, even when the growth process seems, under the microscope, to be leading to cancer, unless the milk factor has been received a limit is set at the stage of hyperplasia. In other words, when breast tissue is examined from mice of the same strain receiving the same dose of oestrogen, some with milk factor and some without, no prophecy as to the possibility of the subsequent development of cancer can be made unless the presence or absence of the milk factor is known. Thus the oestrogen would appear to act as a developing factor upon a breast already sensitised by the milk factor, as described by Mottram (1944) in the case of skin cancer. This idea receives some support from the fact that Orr was able to replace the milk factor with methylcholanthrene and it would harmonise with the observed fact of a continuous growth process, diffuse but not general throughout the gland, the growth proliferation gradually increasing in intensity until the stage of malignancy is reached.

In Cheate's view (Cheate and Cutler, 1931) the first stage in the cancerous process in the human subject is desquamation of the epithelium lining the small terminal ducts and less frequently the acini. He regards the desquamation as the essential cause of cyst formation, the cysts being large in the acini and small in the terminal ducts. Replacement of the desquamated cells demands growth of those remaining, which in turn leads to intraduct neoplasia, and this may be either benign and localised (forming papillomas) or malignant (intraduct carcinoma). Thus cystic disease of the human breast is a new growth or cystoma. In the mouse there are three kinds of cysts:—(1) dilated ducts, which may attain a large size; (2) dilated acini, which are usually smaller; and (3) cysts which result from intra-acinous carcinoma (fig. 1). Desquamation is an uncommon occurrence but may be seen occasionally in dilated ducts and associated with invasion by polymorphonuclear leucocytes. In fig. 16 desquamation is associated with papillary formations.

Lactation can be demonstrated in mouse carcinoma, as in fig. 17. It was seen by Dawson (1935a) in a human adenoma and again (1935b) in a canine papilloma. Carcinoma can be seen to arise in lactating acini (fig. 15), the aberrant and anaplastic cancer cells still forming a lining to the acini. This particular change occurred in a breast the seat of a well developed cancer in a mouse in which there were similar tumours in two other breasts. All the carcinomata contained lactating areas. I have not been able to find any description of lactation in a human breast cancer. Lactation can not infrequently be seen in the proliferating groups of acini occurring in oestrogen-treated male mice.

Other conditions common in the human subject have been searched for but not found in the mouse. (1) *Fibroadenoma*. In the mouse, cystic adenomas are not uncommon (fig. 10) and may be surrounded and interwoven with fairly dense connective tissue. But the characteristic whorling of fibrous tissue and the sharp definition

## MAMMARY CANCER OF MOUSE



FIG 15—RIII female breeder, aged 49 weeks. Malignant acinous proliferation in lactating acini  $\times 200$

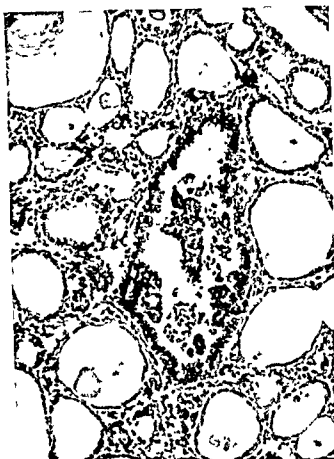


FIG 16—IFS female breeder, given milk factor by RIII, aged  $14\frac{1}{2}$  months. Central part of a mixed tumour consisting of benign cystic areas, malignant acinous proliferation and acini such as that seen in the centre of the figure. This contains desquamated epithelial cells and papillomata covered with hyperplastic epithelium. A very unusual type of breast tumour.  $\times 150$ .

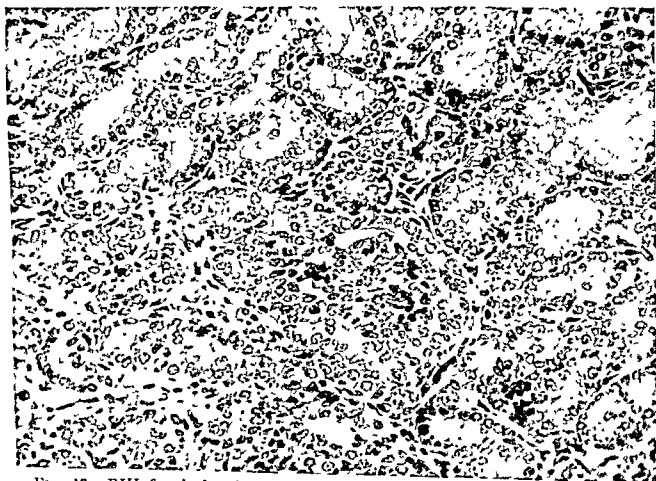


FIG 17—RIII female breeder, aged  $6\frac{1}{2}$  months, at end of lactation. Mammary carcinoma showing lactating acini.  $\times 335$





of the human intracanalicular fibroadenoma do not occur. (2) *Paget's disease of the nipple*. From time to time the convergence of main ducts to the nipple is seen in female mice, but in the absence of serial sections the nipple itself rarely appears. In the male mouse there is no nipple (Orr). Thus the chances of observing intra-epithelial spread of cancer in the nipple and surrounding skin are not very great. But no such spread has been seen in large ducts nor acini and thus it would seem probable that it is of infrequent occurrence. The mode of dissemination of cancer in the mouse is different from that in man, as metastasis takes place via the blood stream rather than via the lymphatic system. (3) *Pink-cell cystoma*. Nothing resembling this well recognised human structure has been seen in the mouse.

### SUMMARY

1. In a study of the evolution of mammary cancer in the mouse, intra-acinous carcinoma was the first and most frequent form of cancer to be observed and was accompanied in a small number of cases by intraduct carcinoma. Lumination is a notable feature of this change, resulting in the formation of groups of tumour acini within the walls of the original acinus. Malignant acinous proliferation also occurs (fig. 8), such areas being associated with intra-acinous carcinoma to form a palpable tumour.

2. A study of the effect of the addition or withdrawal of the milk factor has shown it to act mainly upon the acini, leading to a high degree of acinous proliferation and intra-acinous and extra-acinous carcinoma. There is much variation in the degree of acinous proliferation preceding the initiation of the malignant process but in general the high degrees of hyperplasia lead more frequently to cancer. Extra-acinous or infiltrating carcinoma can be shown in nearly all cases to be preceded by intra-acinous carcinoma.

3. In mice which have received oestrogen but not the milk factor, a limit is set upon the growth process at the stage of hyperplasia. Thus the oestrogen would appear to act as a developing factor upon breast tissue already sensitised by the milk factor.

4. There is a difference in the site of origin of mammary cancer in the mouse and in man, where intraduct cancer (either as a sequel to papilloma or apart from this condition) is far more common than primary intra-acinous cancer. Large or small ducts may be affected, but more usually the terminal ducts. This observation raises the question as to whether a milk factor exists in the human subject and, if so, whether it is of a different kind, acting upon the ducts.

5. There are many points of similarity in the evolution of cancer in the two species; in both the origin is frequently multicentric and in both the process is often a gradual one, to be seen at different stages in the same breast or in different breasts in the same subject. It is not possible to state exactly at what stage cells acquire the



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1 mg. uric acid, about 2 mg. guanine. None gave a reticulocyte response.

4. *Iron*. Two hundred mg. of iron and ammonium citrate in 10 c.c. of saline injected intravenously into a rabbit produced a reticulocyte peak of 5.2 per cent. on the sixth day. This is a very large dose and iron is not present in such quantities in the liver preparations. Twenty mg. of iron and ammonium citrate failed to produce any reticulocyte response in two splenectomised rabbits.

It was considered reasonable to rule out all these impurities as the cause of the reticulocyte response to liver extracts.

### B. Fractionated liver extracts of unknown potency

In view of these results, the following liver fractions prepared by Dr Neuburger were tested.

*Extract T<sub>4</sub> (unfiltered)*. In two different rabbits 5 mg. of extract gave reticulocyte peaks of 7.1 per cent. (fig. 1, D.1) and 8.8 per cent. respectively. Doses of 1 mg. produced peaks of 7.0 and 6.3 per cent. on two injections (fig. 1, D.2). 0.5 mg. gave a peak of 5.0 per cent. (fig. 1, D.3). It will be noted in fig. 1 that another

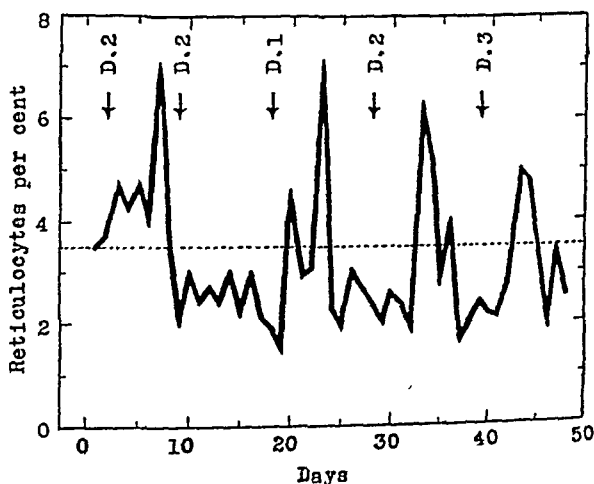


FIG. 1.—Reticulocyte response of splenectomised rabbit to the injection of the fractionated liver extract T<sub>4</sub>.

D.1 = 5.0 mg. of T<sub>4</sub> (unfiltered) injected intramuscularly.

D.2 = 1.0 " " " " "

D.3 = 0.5 " " " " "

..... = upper limit of spontaneous variation.

injection of 1 mg., given two days after the first peak, failed to produce a response, as the bone marrow was in a "refractory" phase. Five mg. of fraction T<sub>1</sub> tested clinically gave a good response in a case of pernicious anaemia.

*Extract F11*. Five mg. of this fraction gave a reticulocyte peak of 7.0 per cent. in a rabbit (fig. 2, E.2). The same dose sterilised by a

Seitz filter gave a peak of 4.0 per cent. only (fig. 2, E.1). This was explained by the results of a nitrogen analysis, when it was found that the filtrate contained only 8 per cent. of the nitrogen of the unfiltered material. Seventeen mg. of this extract injected intra-

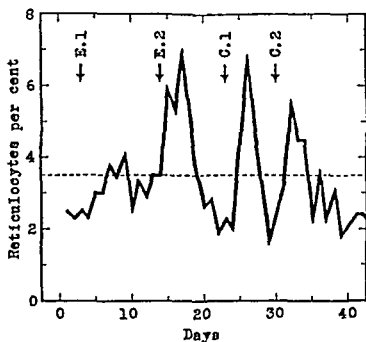


FIG. 2.—Reticulocyte response of splenectomised rabbit to the injection of (E) the fractionated liver extract F.11 and (C) a 70 per cent. alcohol-soluble, absolute alcohol-insoluble fraction of the crypts of Lieberkühn.

E.1 = 5 mg. of F.11 (filtered) injected intramuscularly.

E.2 = 5 " " (unfiltered) injected intravenously.

C.1 = 3 " of an extract of the crypts of Lieberkühn injected intravenously.

C.2 = 6 " " " " " "

muscularly into a patient with pernicious anæmia caused a reticulocyte percentage rise from 0.5 before injection to 14.0 on the 5th day after injection. Hb rose from 55 to 76 per cent., R.B.C. from 2,200,000 to 3,490,000 per c.mm. within eight days.

*Extract N.* 11.5 mg. injected into three rabbits gave reticulocyte peaks of 4.8 per cent. (fig. 3, N), 3.9 and 4.1 per cent., all of which fall below a maximal response. Tested clinically, 66.5 mg. of this extract given in three divided doses produced a rise of R.B.C. from 2.5 to 3.3 million within five weeks. None of the doses produced a maximal reticulocyte response, confirming the result of the rabbit test and showing that this fraction was not as potent as the preceding ones.

Two liver fractions (15V<sub>3</sub> and 34B) were inactive, both clinically and in the splenectomised animal. Four other fractions were found to be inactive in the animal test but were not tried clinically.

### C. Intestinal extracts

As the villi and crypts of Lieberkühn of the small intestine show hæmopoietic activity when given by mouth to patients with pernicious

kindly undertook the synthesis. A dose of 0.8 mg. injected into a splenectomised rabbit gave a reticulocyte peak of 4.2 per cent. on the tenth day (fig. 3, L.1). A dose of 2.5 mg. administered by stomach tube to two rabbits also gave a series of reticulocyte peaks. In the first rabbit a peak (5.5 per cent.) occurred on the third day (fig. 3, L.2); in the second, on the second day (4.4 per cent.), followed by a peak of 4.0 per cent. on the fifth day.

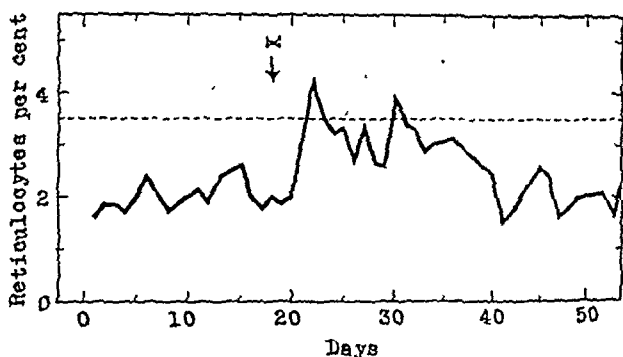


FIG. 5.—Reticulocyte response of splenectomised rabbit to the intramuscular injection of 0.2 mg. of synthetic xanthopterin.

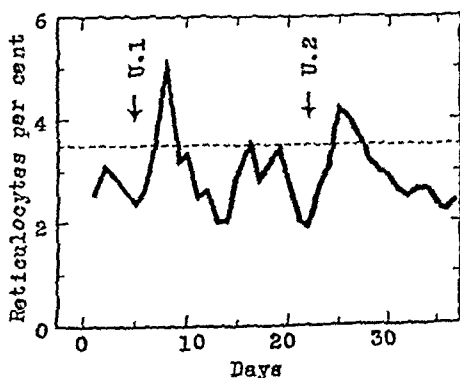


FIG. 6.—Reticulocyte response of splenectomised rabbit to the intravenous injection of a fused uric acid pigment.

U.1 = 10 mg. injected (unfiltered).

U.2 = 10 „ „ (filtered).

*Xanthopterin.* Crude xanthopterin was kindly prepared by Dr Lythgoe (Department of Chemistry, Cambridge University) from the wings of butterflies. One mg. of this preparation injected intravenously into a rabbit produced three reticulocyte peaks of 4.1, 4.3 and 4.9 per cent. on the 5th, 11th and 13th day respectively.

Synthetic xanthopterin was very kindly prepared by Dr Quibell and Dr F. G. Mann (Department of Chemistry, Cambridge University). Slight activity was shown in doses of 2 mg. injected intramuscularly (fig. 5, X), while 6 mg. injected intravenously into two splenectomised

rabbits produced two reticulocyte peaks in each case—3·8 per cent. on the 4th and 8th days in one rabbit and 4·0 per cent. and 4·1 per cent. on the 3rd and 8th day respectively in another rabbit.

*Fused uric acid pigment.* This preparation was made available through the kindness of Dr F. G. Mann. A dose of 10 mg. of his material, which still contained a number of by-products of the reaction in addition to the pigment, produced a reticulocyte peak of 5·0 per cent. on the 3rd day in a splenectomised rabbit (fig. 6, U.1). This dose was given intravenously and was not passed through a Seitz sterilising filter. Subsequent intravenous injections into two rabbits of 10 mg. filtered through a Seitz sterilising filter gave peaks of 4·2 and 3·8 per cent. respectively on the 3rd day (fig. 6, U.2). A considerable amount of pigment was retained on the filter pad. A preparation containing less xanthopterin gave the following results:—20 mg. given intravenously produced peaks of 3·5 and 4·0 per cent. respectively on the 3rd day in two rabbits, while 30·35 mg. given intravenously produced peaks of 4·1 and 4·2 per cent. in two rabbits.

### DISCUSSION

In a previous paper it was suggested that the spleen exerts a regulating influence on the erythropoietic activity of the bone marrow. Thus, after splenectomy, the marrow will respond to a hæmopoietic stimulus such as the injection of a potent liver extract. It might be argued that the bone marrow without the regulating influence of the spleen would be capable of responding to other known hæmopoietic stimuli, *e.g.* ascorbic acid or iron. These substances were tested so as to exclude any possible interference from traces which might be present in the liver extracts used. As described above, doses of 10 mg. of ascorbic acid and 20 mg. of iron and ammonium citrate given intravenously failed to produce any response, but it is possible that very large doses of substances other than the anti-pernicious anæmia factor may produce a reticulocyte response (*e.g.* iron, p. 424). Impurities in liver extracts cannot be the cause of the reticulocyte response, as these substances are present in greater abundance in the less purified and less potent extracts. Also the concentration of impurities would vary considerably in the less purified extracts, depending on the different methods of preparation.

#### *Localisation of the anti-pernicious anæmia factor in the gastro-intestinal tract*

The anti-pernicious anæmia substance has been found in nearly all parts of the gastro-intestinal tract. That the stomach cannot be the only place where it is elaborated is clearly shown by the fact that only very rarely does total gastrectomy in man cause pernicious anæmia (Jones, 1940; Joll and Adler, 1942; Doehring and Eusterman,



1942; Farris *et al.*, 1943), nor does total gastrectomy in animals, including monkeys, have this effect (Ivy, 1940). Even more extensive resections of the alimentary tract have failed to do so.

The only factor common to the various regions of the alimentary canal which have been shown to be therapeutically active is the occurrence of argentaffine cells. The layer of the mucosa which contains them is also the layer which exhibits therapeutic activity. Thus the Brunner's glands of the duodenum are almost free from argentaffine cells and are therapeutically inactive. The argentaffine cells are considerably reduced in number in cases of pernicious anæmia (Jacobson, 1939). In short, these cells appear to be the carriers of the therapeutic activity of the mucous membrane.

Magnus (1940) points out that argentaffine cells occur in the metaplastic islands of intestinal mucosa which can be found in the atrophic mucous membrane of the fundus of the stomach in cases of pernicious anæmia. This, however, does not exclude the possibility of the argentaffine cells playing an important part in normal erythropoiesis, since in pernicious anæmia the areas of the gastric mucosa which show intestinal metaplasia with argentaffine cells are small compared with the whole area of the gastric mucosa. Even severe cases of pernicious anæmia still form some red blood cells.

### *The chemical nature of the anti-pernicious anæmia factor*

This is still undetermined. Highly purified liver preparations have been investigated by Dakin *et al.* (1936), Laland and Klem (1936), Jacobson and Subbarow (1937, 1941), Mazza and Penati (1937-38), Karrer *et al.* (1938), Dakin and West (1939), Karrer (1941), Erdos (1942) and Mazza (1942). The subject has been summarised by Jacobson and Subbarow (1941). When these data are examined only a few positive and common findings emerge. The following amino acids were absent in one or more of the four preparations of Dakin and of Karrer:—glycine, phenylalanin, tyrosin, tryptophane, prolin, hydroxyprolin, histidin and lysin.

In 1943, however, Karrer and Keller supplemented the analysis of Karrer's (1941) liver preparation. It contained arginin, histidin (absent from the 1938 fraction), lysin (absent from the preparation used by Dakin *et al.* (1936)), and the following  $\alpha$ -amino acids:—leucin, valin, alanin (no  $\alpha$ -amino acids were found in the preparation used by Karrer *et al.* (1938), which was clinically tested by Koller (1938)) and finally glutamic acid. The only other analysis in which glutamic acid is mentioned is that of Dakin *et al.*, where it was found with aspartic acid. With the incomplete data available so far, arginin is the only amino acid that has been tested for and found in all preparations active against pernicious anæmia. The sulphur-containing amino acids can also be excluded from the list, as Dakin and West's extract was free from sulphur. There is little information available

as to the presence of other constituents Phosphorus, purines and nicotinic acid were not found in Dakin and West's preparation, but in view of the facts recorded in this and a previous paper (Jacobson, 1939), it is of particular interest to discuss the role pterins may play in normal erythropoiesis

Tschesche and Wolf (1936) reported that the injection of 10  $\gamma$  xanthopterin caused considerable new formation of red blood corpuscles in rats made anæmic by feeding with goat's milk. In 1937 they reported that when such rats were treated by daily intramuscular injections of 0.5  $\gamma$  xanthopterin the further progress of the anæmia was arrested

Simmons and Norris (1941) cured a fish anæmia by injecting 30.50  $\gamma$  xanthopterin into the anæmic fish

Totter *et al* (1944) found that xanthopterin was partially effective in the nutritional anæmia of monkeys kept on a vitamin M deficient diet. One mg xanthopterin given daily delayed the onset of the anæmia from the third till the ninth month. These experiments show that xanthopterin is one of the requirements for normal red cell formation, but this nutritional anæmia is fully cured by another factor (vitamin M) present in yeast and liver

McKibbin *et al* (1942) found that a uropterin (xanthopterin) preparation made from 100 g of dried liver, given daily to dogs made anæmic by bleeding, accelerated the rate of hæmoglobin and red cell formation. The rate of regeneration was about half that produced by feeding 50 g of dried liver daily

These observations all suggest that pterins play a part in erythropoiesis

With regard to the presence of pterins in the liver extracts, B. M. Jacobson and Subbarow (1937) found a complex purin (pterin) and Mazza and Penati (1937-38) also found a pterin. Other authors do not indicate whether or not pterins were present, but Karrer *et al* (1938) and Karrer (1941) state that there were no pterins present in their liver extracts, they do not describe a method of testing for these substances. Here it may be mentioned that a negative murexide reaction is not sufficient to establish the absence of a pterin. Whether the gastro intestinal principle and the liver principle are chemically identical is not known, but in this connection it is interesting to note that the argentaffine cells of the gastro intestinal tract contain xanthopterin (Jacobson, 1939, Jacobson and Simpson, 1946)

Castle *et al* (1944) have shown that xanthopterin cannot be the extrinsic factor, as gastric juice when incubated with xanthopterin does not produce a reticulocyte response in patients with pernicious anæmia

The presence of pterins in preparations of liver and intestine and the activity of xanthopterin and leucopterin in splenectomised rabbits does not mean that pterins are identical with the anti pernicious anæmia factor, but it strongly suggests that pterins are a constituent of the active principle

## SUMMARY

1. The reticulocyte response of the splenectomised rabbit was used to assay the anti-pernicious anæmia activity of purified liver extracts.

2. Injections of extracts of crypts and villi of the small intestine and of extracts of an argentaffine cell tumour (carcinoid) were similarly active in these animals.

3. Clinically inactive liver extracts as well as a number of non-specific substances failed to produce a response.

4. Synthetic xanthopterin, leucopterin and the pigment derived from fused uric acid gave a reticulocyte response in the splenectomised rabbit.

The authors' grateful thanks are due to Dr Honor B. Fell and Sir Frederick Hopkins for their interest and help in this work. They are indebted to Dr T. Quibell, Dr F. G. Mann and Dr B. Lythgoe for the supply of pterins and to Dr Neuberger for liver extracts. Their thanks are due to Professor A. R. Todd for his interest and to the honorary physicians of Addenbrook's Hospital for their helpful co-operation. They also thank Mr V. C. Norfield for preparing the figures. The expenses of the investigation were defrayed by the Medical Research Council and the Lady Tata Memorial trust.

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## SUMMARY

1. The reticulocyte response of the splenectomised rabbit was used to assay the anti-pernicious anaemia activity of purified liver extracts.

2. Injections of extracts of crypts and villi of the small intestine and of extracts of an argentaffine cell tumour (carcinoid) were similarly active in these animals.

3. Clinically inactive liver extracts as well as a number of non-specific substances failed to produce a response.

4. Synthetic xanthopterin, leucopterin and the pigment derived from fused uric acid gave a reticulocyte response in the splenectomised rabbit.

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# ARRHENOBLASTOMA: REPORT OF A CASE WITH UNUSUAL FEATURES

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(PLATE LX)

TESTICULAR tumours of the ovary are sufficiently rare to warrant the publication of another case

## *Clinical history*

F C, a female aged 61, was admitted to hospital on 8.8.42. There was no relevant family history. Her only (elder) brother was in good health and had had a family of three, all normal. Her own health had been good except for amenorrhoea and sterility. She had never experienced any menopausal phenomena. She married at the age of 29 and had had a normal degree of libido. Heterosexual desires only had been experienced. At 18 she had transient discomfort in the left groin, diagnosed by the family doctor as "weakness" and not requiring treatment. In 1932 this old complaint, which had only troubled her occasionally in the previous three years, caused her to consult a surgeon who performed an operation for left inguinal hernia. A lump of tissue found in the inguinal canal proved on microscopical examination to be cryptorchid testis. No further details were available. Shortly after her recovery she complained of weakness in the right groin and was advised to wear a truss. In 1938 she began to have pain in the right iliac fossa and to lose weight. She volunteered the information that the most manifest loss of flesh was the disappearance of fat around the shoulders and in the breasts. She consulted her doctor, who found a tumour the size of a coconut in the right iliac fossa. He sent her with a letter, written in 1939, to the Royal South Hants Hospital, but she did not deliver it until 8th August 1942.

*Clinical findings* (10.8.1942). Patient was of medium height and well built. The secondary feminine characteristics appeared normal. The external genital organs were hypoplastic. There was no hirsuties and the distribution of the hair on the pubis was feminine. The voice was high pitched and the breasts somewhat hard and shotty but of normal size. There was a firm smooth swelling rising out of the pelvis, with a softer superimposed swelling on the left side. There was a bilateral inguinal hernia (direct recurrence) on the left side. The vagina was 2.5 cm. long and had a smooth and regular surface, with no dimple or protuberance to denote rudimentary parts of a uterus.

*Laparotomy* (17.8.42). A retroperitoneal tumour was removed apparently intact. It was of large size and extended from the pelvis to above the umbilicus. It was mostly solid but showed central necrosis and cystic change. Absence of the uterus was noted.

*After history*. The patient made a good recovery and was kept under observation. In March 1943 a hard tender mass the size of a large orange

was palpated in the right upper abdomen. It was not attached to the liver but was deeply rooted in the posterior wall. Palliative deep X-ray treatment caused considerable reduction in size but not its complete disappearance. The general condition of the patient improved and she was symptom-free until September 1943. Subsequently a bilateral pleural effusion, an irregularly enlarged liver and a hard mass in the left supraclavicular region were found. She died on 17th April 1944.

### *Post-mortem findings*

The body was emaciated: otherwise the external appearances were normal for the supposed age and sex. The vagina was a blind sac and there was no trace of cervix or uterus. The pouch of Douglas and pelvis generally were lined by smooth peritoneum. There was a huge mass of new growth in the pre-aortic lymph glands, extending to a depth of about 15 cm. On section this showed brain-like growth with many areas of necrosis and hæmorrhage and numerous cysts up to 2.5 cm. in diameter filled with mucoid fluid.

The liver weighed 80 oz. It contained one large cystic growth 5.0 cm. diameter and about a dozen smaller ones. The right adrenal contained a fibrous nodule: the left appeared to have been destroyed by the surrounding growth. The kidneys, spleen, stomach and intestines were normal. The pleural cavities each contained a pint of clear fluid. On the right side of the spine behind the pleura there were many glands full of growth up to 2.5 cm. diameter. These had eroded the bone and were continuous with a large tumour mass behind the lower lobe of the right lung, measuring 17 cm. vertically by 20 cm. horizontally, by 10 cm. antero-posteriorly, extending from the right dome of the diaphragm to the bifurcation of the trachea. Section showed new growth of a dull red colour, again containing necrotic tissue and cysts. There was another mass the size of an orange in the left supraclavicular fossa. There was no growth to the left of the spine. The right lung showed numerous plaques of growth all over the pleural surface, confluent at the apex, discrete elsewhere. The left lung was free from growth. Heart, brain and other organs showed nothing of significance.

### *Histology*

*Biopsy specimen, 17.8.42.* The structure is that of a Sertoli cell tumour of testis (figs. 1 and 2). Ewing (1940) pictures a tumour of similar appearance (fig. 316) which he describes as a testicular tumour of the ovary. It shows fairly regular tubules lined by 3 or 4 layers of cells with their oval nuclei lying at right angles to the basement membrane and giving the characteristic palisade appearance described by previous observers. The cell outlines are indistinct. In each tubule there is an apparent lumen filled with granular or hyaline secretion. No mitoses nor giant cells are seen. Stroma is scanty.

## ARRHENOBLASTOMA



FIG 1.—Arrhenoblastoma. Low-power view of primary ovarian tumour.  $\times 85$ .

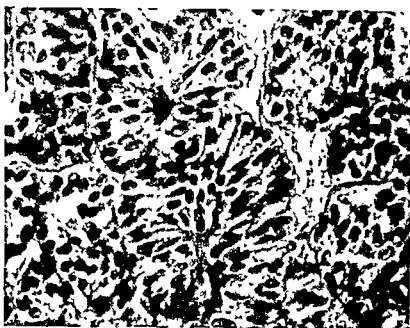


FIG 2—High power view of primary tumour.  $\times 390$ .

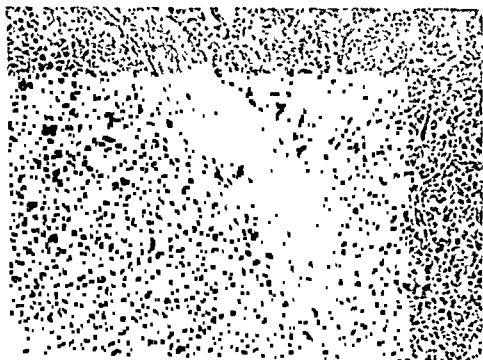


FIG. 3.—Arrhenoblastoma. Secondary deposit in liver. The tumour has now assumed a sarcomatous aspect.  $\times 100$ .





the tubules being closely packed but separated by a delicate well defined basement membrane. No testicular interstitial cells are seen.

*Post mortem material* (1) *Glands and lung* Here the tumour is composed of small elongated cells with scanty cytoplasm and indistinct outline. The nuclei are oval and vesicular, with no distinct nucleolus. The cells lie loosely and irregularly like those of a sarcoma, but in places they form short columns and imperfect tubules. There are large cystic spaces containing albuminous fluid and lined by the tumour cells. They do not appear to be the result of degeneration. The blood vessels are capillaries, with a single layer of endothelium. Reticulum is scanty and does not form a defined pattern.

(2) *Liver* The secondary deposits here show in part the structure described above but in some areas the tumour resembles a pleomorphic sarcoma (fig 3). The cells are rounded or elongated and of varying size, with distinct outlines, the nuclei are large and vesicular. The cells are often multinucleated, of malignant giant cell type, and mitotic figures are numerous. The stroma is very scanty. There is no fibrosis at the advancing edge of the growth, which infiltrates directly among the liver cells. Reticulum fibres accompany the fine blood vessels but do not spread out between the tumour cells.

It is thus seen that while the original tumour shows a well differentiated tubular structure the metastases are highly anaplastic and have a sarcomatous appearance.

### Discussion

Tumours of this type have been found in both testis and ovary, in which they were described by Pick (1905). They are uncommon tumours. Kruckman (quoted by Innes, 1942) states that the tumour has been recorded in ectopic testes (16 cases) in the ovaries of women showing menstrual disturbances but without other sexual disorders (3 cases) and in pseudohermaphrodites (16 cases).

In the ovary these tumours are classed as arrhenoblastomas. This neoplasm usually occurs in a previously normal woman and causes, apart from symptoms ascribable to its mere presence in the pelvis (sometimes negligible), a cohort of symptoms and signs of defeminisation and masculinisation. Amenorrhœa, sterility and atrophy of the breasts, endometrium and genital organs (with the exception of the clitoris, which frequently shows signs of hypertrophy) belong to the negative defeminising signs. Hirsuties, loss of weight due to the disappearance of fat deposits in the hips and breasts causing inversion of the feminine pelvis—shoulder ratio, enlargement of the larynx with the production of a lower pitch of voice are positive signs of masculinisation and do not always make their appearance. These signs and symptoms disappear directly the tumour has been surgically removed and even pregnancy with delivery of a full term child has been recorded. The clinical features

mentioned above return when recurrence takes place or disappear only partially and temporarily when removal is incomplete or when peritoneal dissemination or more wide-spread metastasis has already occurred.

The highest incidence is in the fourth decade—range 16-66. The course of the disease is slow but progressive, and the tumour, if not removed or treated with combined surgery and radiation, causes death with metastases in the pelvis, lymph nodes, liver, lungs and mediastinum (Ewing, 1940).

Arrhenoblastoma is a rare tumour, though no doubt more common than the 54 cases reported to date would indicate. Except for the case of Bell (1914-15) in England and McIntyre (1942) in Australia no cases have been recorded in the English list. Norris (1938), out of 37 published cases, considers only 28 as acceptable, as he is strongly of the opinion that a pathological diagnosis of arrhenoblastoma is impossible and that only the association of the appropriate clinical and pathological findings justify the use of this term. That clinical features also may lead astray is shown by the case of arrhenoblastoma published by Norris himself in 1938 and which four years previously had been diagnosed and published as a case of adrenal cortical hyperplasia (Walters *et al.*, 1934).

Burrows (1943) suggests the use of the term arrhenoma (male tumour—Ewing), which he would apply "to all tumours, whether situated in the ovary, adrenal or elsewhere, which produce excessive amounts of androgen and so bring about virilism".

R. Meyer's (1931) subdivision of arrhenoblastoma into two morphological forms and one intermediate type based on a score of his own cases seems to offer a working basis which may be corrected and adjusted in the future as more cases are reported. This author coined the term arrhenoblastoma and distinguishes (1) adenoma tubulare testicularis, with a microscopical appearance of embryonic testis, which only rarely causes masculinisation; (2) an atypical group with a sarcoma-like structure, rudimentary cords and irregular tubules; this type of tumour is always associated with the most manifest masculine changes; (3) an intermediate group, with portions resembling groups 1 and 2 and clinical manifestations slighter than in group 2. Meyer does not consider the presence of defeminising or masculinising signs as a *sine qua non* for a diagnosis of arrhenoblastoma. Only 3 out of 9 cases of group 1 in Meyer's collection (1931) presented these signs. On the other hand Phelan's (1934) case conformed to the morphology of group 1 and had an obvious masculinising syndrome, while Goodall's (1934) group of 3 cases had no masculinising signs at all.

The problems of nomenclature and assessment of diagnostic and histological criteria are linked up with that of pathogenesis. Pick assumed an origin from an ovotestis. Meyer (p. 709) on the other hand believes they originate from undifferentiated germ cells which

retain their sexual potency. Under certain external conditions "they begin late in life to proliferate and then only begin to exert an influence in the direction towards maleness". In the hilum of the ovary the blastema produces the rete ovarii and some medullary cords or tubules which are the homologues of the rete testis and the tubuli efferentes of the testes. The rete ovarii and medullary tubules have bisexual potentialities, but it is only when they proliferate and produce a tumour that they exert a hormonal male-directing influence.

Wilfred Shaw (1942, p. 289) says "It may be that some of the cells in the ovary are in a state of dynamic equilibrium, so that the dividing cells may revert back to the structure of ancestral cells of the foetal epoch". For Norris (p. 28) there seems no reason, morphological or genetic, for deriving this neoplasm from any particular part of the primitive sex gland. The arrhenoblastoma "should be looked upon as a malignant ovarian tumor whose morphologic picture more or less corresponds to certain, some, or all of the varying structural conditions found in the indifferent stage of the sex gland's development".

Novak and Long (1933, p. 1060) consider the rete ovarii an actual homologue of the male testis. Every woman according to these authors shelters a potential testis. "Under certain conditions this undifferentiated male tissue may become active, and its male endocrine influence may override the primary female tendency, with the production of various degrees of intersexuality".

The diagnosis of arrhenoblastoma in the case here reported has been made on the histological findings and the clinical features in regard to the history and progress of the disease. In the differential diagnosis, in the absence of manifest masculinising signs, other clinical syndromes which usually have to be taken into consideration like Cushing's syndrome, adrenal cortical tumour, diabetes of bearded women etc. have been discounted. Only one ovarian tumour—the dysgerminoma—had to be taken into account. The 72 cases of dysgerminoma collected by Novak and Gray (1938) occurred mostly in pseudo- or true hermaphrodites. The clinical diagnosis of dysgerminoma presented itself therefore with some strength, but the histological findings were those of a testicular tubular adenoma and could not be confused with those of dysgerminoma. It would be very difficult and indeed futile to attempt to establish the position of our patient as regards sex. The finding of a testicle at the operation twelve years previously, associated with absence of the uterus and hypoplasia of the external genital organs, together with absolute amenorrhœa, places the patient, who otherwise exhibited normal secondary feminine characteristics, in the class of intersexuals. The sequence of events in the patient's development and subsequent pathological abnormalities is obscure and any assumption in this respect would be purely hypothetical. It is impossible to say whether

the intersexual condition preceded the arrhenoblastoma or whether it followed the establishment of the growth, which remained slow-growing and quiescent for many years, possibly since before puberty, and gave rise to symptoms only later in life.

The cases of arrhenoblastoma so far recorded do not warrant any definite conclusion as to the pathogenesis of this tumour.

*Malignancy.* The progressive course, the recurrence in cases of late or incomplete removal and the metastatic dissemination dispel any doubt as to the malignancy of the testicular adenoma of the ovary, considered by some authors as relatively benign. A fatal termination has already been recorded in the cases of Norris and Novak and Long and recurrences after many years in several others.

Post-operative radiotherapy is advocated by several authors; others are against irradiation in view of the danger of affecting the other ovary and so preventing refeminisation. In our view post-operative radiation treatment should be approached on the lines followed when dealing with other malignant tumours of the ovary. In patients under thirty-five the points to be considered are whether the tumour has been removed in its entirety and intact, its size, probable duration and clinical features.

### Summary

A case of arrhenoblastoma of the ovary with intersexuality of at least 7 years' duration (possibly 40 years since puberty) is described and discussed. The tumour recurred after removal and ultimately disseminated widely.

We are indebted to Mr H. J. Nightingale, M.S., F.R.C.S., senior honorary surgeon to the Royal South Hants and Southampton Hospital, both for permission to publish this case and for helpful criticism.

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## THE HISTOGENESIS OF EXPERIMENTAL PNEUMONIA IN MICE

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(PLATES LXI LXV)

ANDREWES, Laidlaw and Smith (1934) showed that pulmonary lesions could be produced in mice by intranasal inoculation under ether anaesthesia with the viruses of human and swine influenza. They thought that such lesions were readily produced by these viruses only, and that bacteria played little or no part in their development. However, as a result of the work of Neufeld and Kuhn (1934-35) and of Hoyle (1935) it became clear that inflammatory lesions of the lung could be readily produced with almost any mouse pathogenic agent, and the technique is now widely used in the study of mouse pathogenic bacteria, protozoa, rickettsiae and viruses. Hoyle thought that there was little essential difference between bacterial and virus lesions, but Straub (1937, 1940) demonstrated that the influenza viruses produced a characteristic lesion of the bronchial epithelium.

The present paper describes a more detailed study of the histology of the bacterial lesions and confirms Straub's work.

### EXPERIMENTAL PROCEDURES

The mice were inoculated intranasally under light ether anaesthesia with 0.05 to 0.1 ml. of culture or lung extract. Animals were killed at intervals or examined when they died. Cultures were made from lung and systemic blood. Unless the distribution of lesions made modification desirable, the left lung was used for cultures, while the right lung was fixed intact in 4 per cent formaldehyde saline and the paraffin block cut down to the plane of maximal area. Sections from this region were stained with haemalum and eosin, iron haematoxylin and van Gieson and Lawson's elastic stain, and by Gram's method or carbolfuchsin for organisms. Sections of trachea were examined in many instances and other organs in a sample of each group.

### RESULTS

It was evident that infective material was not held up in the bronchi but was reaching the alveoli in a very short time after inoculation. We inoculated a small group of mice with a suspension of ferric ferrocyanide by the same method and killed the mice at intervals. In those killed immediately, a large part of the Prussian blue had entered the oesophagus and stomach, in the respiratory

tract it was present in the trachea and main bronchi. After five minutes it was in the substance of the lung and in ten minutes patches were clearly visible under the visceral pleura; it was most abundant in the ventral and cephalic parts of the lung, presumably a hypostatic effect. Microscopically, the bulk was in groups of alveoli which were more or less collapsed, though there was still a thin layer on the surface of the bronchial epithelium (fig. 1).

The bacteria used varied greatly in pathogenicity, from those which killed every animal in an experiment to those with which all survived, but even with the latter some evidence of inflammatory reaction was found in the lungs. When infective material first reaches the alveoli, all lobules are not affected, the primary lesions being focally distributed. In such foci the alveoli are more or less collapsed and contain polymorphs and mononuclear cells, the proportion of polymorphs being higher with the more virulent bacteria. This initial stage we shall refer to as "collapse inflammation". Sometimes the process takes the form of trabeculae of collapse dividing groups of normal alveoli ("trabecular collapse inflammation") (figs. 2-5).

The stage of collapse inflammation is found in some degree with all bacteria. In the case of non-virulent bacteria it is followed by resolution, the exudate gradually loosening and the alveoli reopening; during the process large active macrophages containing cellular debris etc. are seen (figs. 6 and 7). In the more severe infections, inflammation seems to spread in two main ways: (1) by progressive extension from the periphery of individual primary foci to the alveoli surrounding them (figs. 8 and 9), ultimately resulting in massive consolidation by confluence; (2) along the adventitia of the vessels and bronchi through a considerable tract of lung (figs. 10-12). There has been little evidence of the spread of infection up and down the lumina of bronchi.

The second method of spread seems to be of great importance in the development of extensive fatal pneumonia, especially those lesions most like human lobar pneumonia. The vascular adventitia in the primary foci of collapse inflammation becomes swollen, oedematous, congested or actually hæmorrhagic and later infiltrated with leucocytes. Inflammation may then track along it, extending beyond the primary focus and along the vascular tree into other parts of the lung where there is as yet no evidence of alveolar involvement. This process is much more marked round blood vessels than bronchi. Whether arteries or veins are involved depends on the distribution of the primary lesions. The branches of the arteries accompany those of the bronchi throughout the lung, so that they may be implicated in quite small peripheral foci of collapse inflammation. The veins run independently except in the hilar region, so that central or large peripheral lesions are necessary.

Many mice die at the stage of widespread pulmonary periangitis without further alveolar involvement. In infections of low virulence,

## HISTOGENESIS OF EXPERIMENTAL PNEUMONIA



FIG. 1.—Lung of mouse killed 10 minutes after intranasal instillation of a suspension of ferric ferrocyanide. The Prussian blue has reached groups of alveoli which show various degrees of collapse. There is also a thin layer on the surface of the bronchial epithelium.  $\times 110$ .



**3 days.** Eleven mice killed, 3 dead. Macroscopically, conspicuous patchy congestion. Histologically, like 2-day changes, but mononuclear/polymorph ratio increased; associated bronchioles and atria dilated; œdema. Inflammation of hilar interstitium and tracheo-bronchial lymph glands. Pleural endothelium swollen, with in one case superficial fibrino-leucocytic exudate. Adventitial inflammation occasionally for quite a short distance beyond the primary foci, veins showing mainly mononuclears, arteries also polymorphs and œdema. In the fatal cases, evidence of centrifugal spread of inflammation from the foci of collapse inflammation to the surrounding alveoli; polymorphs relatively more numerous in these mice. Cultures: lungs + in all fatal cases and 5 of the 11 non-fatal cases.

**4 days.** Two killed, 1 dead. Macroscopically, conspicuous patchy congestion in 2, only slight changes in 1 (killed) mouse. Histologically, in the killed mice, polymorphs absent from exudate, collapsed alveoli beginning to open out. In the dead mouse, much trabecular collapse inflammation, with confluent pneumonia and mediastinal lymphadenitis; bronchial epithelium degenerate, probably a post-mortem effect. Cultures: lungs + in 2 cases.

**5-10 days.** Twenty-eight mice killed. Gradual recession of changes. Alveoli reopening, exudate diminishing and mainly macrophages (foamy cells and dust cells), with sometimes lymphocytes and polyblasts, polymorphs rare. In one instance, "epithelium" in some of the alveoli. Resolution slower in the vascular adventitia and mediastinal lymphoid tissue than in the alveoli. None completely resolved. Cultures: lung + in 1 mouse (9 days).

### Pneumococcus

Four experiments, 92 mice, 3 strains of pneumococci.

**First strain.** No mice died, 23 killed after periods of 1-10 days. Changes in lungs similar to those with *H. influenza*, but lesions less congested and early exudate contained a higher proportion of polymorphs. Culture: lungs + in 2 out of 4 (1 day), in 1 out of 4 (2 days); blood — in all.

**Second strain.** Two mice died (in 1 and 2 days); 23 killed after 1-9 days. Dead mice showed (1 day) collapse inflammation with beginning confluence in upper lobes, culture + in lungs and blood; (2 days) both lungs solid, collapse inflammation not prominent, œdema and leucocytic infiltration of arterial adventitia throughout, considerable patchy angiogenous pneumonia. Culture: lungs +, blood —. Killed mice: collapse inflammation more extensive than with first strain; adventitial inflammation sometimes extending beyond the primary foci and in later stages polymorphs and polyblasts replaced by lymphocytes; hilar cellulitis and lymphadenitis; from the third day onwards swelling and mucoid degeneration of bronchial epithelium, with desquamation in one case; resolving angiogenous pneumonia once (9 days); patchy "epithelium" in alveoli once (5 days). Culture: lungs + in 1 out of 3 (2 days), 1/4 (3 days), 1/2 (4 days), 1/4 (5 days), remainder —; blood — in all.

**Third strain (type III).** Two experiments (one with diluted culture). Six mice killed after 1 day; 31 and 7 died in 2 and 3 days respectively. Killed mice showed multiple foci of collapse and less numerous bright red non-collapsed foci; 2 showed pleurisy. Microscopically: collapse inflammation, conspicuous inflammation of vascular adventitia (arteries > veins). Periarteritis spreading throughout the lung, the exudate containing numerous pneumococci, with escape of the latter into adjacent patent alveoli, and occasional early angiogenous pneumonia. Pneumonia possibly confluent in one case. Culture: lungs +, blood + in all. Dead mice (2 and 3 days) showed further development of angiogenous pneumonia, with massive patches of consolidation of non-collapsed alveoli. In places, the site of a future patch of consolidation clearly indicated by myriads of pneumococci escaped from inflamed arterial adventitia.

## HISTOGENESIS OF EXPERIMENTAL PNEUMONIA



FIG. 2.—"Collapse inflammation", *Staphylococcus aureus*. Killed 1 day after infection.  $\times 120$ .



FIG. 3.—Collapse inflammation; the terminal bronchioles contain some pus but they

are empty in 1 day.  $\times 200$ .



FIG. 4.—Trabecular collapse inflammation. *Pneumococcus* (avirulent). Killed at 1 day.  $\times 120$ .



FIG. 5.—Massive collapse inflammation; the empty patent bronchi and atria are prominent against the background of collapsed alveoli with leucocytic infiltration. *Pneumococcus* (avirulent). Killed at 1 day.  $\times 60$ .



## HISTOGENESIS OF EXPERIMENTAL PNEUMONIA



FIG. 6—Delayed resolution of a large patch of collapse inflammation *H. influenzae*. Killed at 9 days  $\times 60$

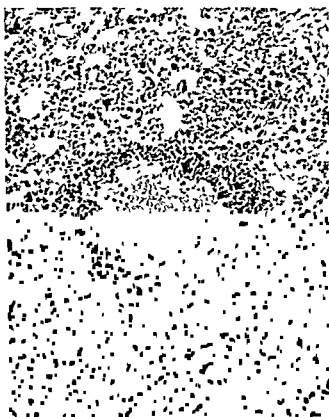


FIG. 7—Stage of resolution of a patch of collapse inflammation, alveoli reopening, polymorphs largely replaced by macrophages, lymphoid infiltration of adventitia of vein. *Pneumococcus* (low virulence). Killed at 9 days  $\times 150$ .

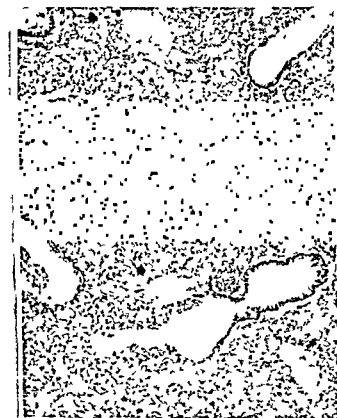


FIG. 8—Pneumonia developing by confluence of the primary lesions of collapse inflammation (cf fig 6) *H. influenzae*. Dead at 4 days  $\times 60$



FIG. 9—Pneumonia by confluence *H. pertussis*. Killed at 4 days  $\times 60$ .



## HISTOPATHESIS OF EXPERIMENTAL PNEUMONIA



FIG. 10.—Collapse inflammation at top of field with bacterial invasion and inflammatory swelling of the adventitia of the associated artery and bronchiole. Extension of inflammation along the arterial adventitia to the lower part of the field where the surrounding alveoli are free from inflammation. Friedländer's bacillus. Dead at 3 days.  $\times 60$ .



FIG. 11.—Widespread extension through lung of adventitial inflammation especially of arteries, surrounding alveoli normal apart from mechanical compression. Pneumococcus. Dead at 2 days.  $\times 40$ .



FIG. 12.—Intense inflammation of an arterial adventitia and early angiogenous pneumonia without collapse of the surrounding alveoli. Pneumococcus. Dead at two days.  $\times 60$ .



FIG. 13.—"Angiogenous" pneumonia showing leucocytic exudate in expanded alveoli. Pneumococcus. Dead at 3 days.  $\times 120$ .



## HISTOGENESIS OF EXPERIMENTAL PNEUMONIA



FIG. 14—Pneumonia  
in to an arterial adventitial lesion  
in upper part of field with collapse inflam-  
mation (vein in midst) in lower part  
Pneumococcus Dead at 2 days  $\times 40$



FIG. 15—Influenza virus A (filtrate) lesion,  
showing degeneration of bronchial epi-  
thelium and leucocytic infiltration of  
bronchial wall, alveoli somewhat emphy-  
sematous, no inflammation Sterile Killed  
at 3 days  $\times 60$



FIG. 16—Bronchiole in upper half of field  
showing virus (filtrate) lesion, portion of an  
artery below Pasteurella and staphylococci  
also present Dead at 6 days  $\times 250$



FIG. 17—Irregular stratification and de-  
generation of bronchial epithelium in virus  
(filtrate) infection Pasteurella also pre-  
sent Dead at 6 days  $\times 250$





No pus in bronchi except in regions of primary collapse inflammation, slight mucoid degeneration of bronchial epithelium. Three day mice showed well marked macroscopic lobar consolidation. Culture all lungs +, blood + in only 1 out of 14 examined, this suggests that death was due to pneumonia and not septicaemia in most cases.

### *Staphylococcus aureus*

Two experiments, 2 strains, 35 mice. Both strains proved avirulent to mice none died. In mice killed at various intervals the early lesions were those of focal collapse without much congestion. Microscopical findings similar to but slighter than those with *H. influenzae*, resolution practically complete by 5 days. Culture lungs + (small number of colonies) after 1 day, — thereafter, blood — throughout.

### *Pasteurella*

As this organism was frequently recovered from the lungs of mice inoculated with influenza virus, it was necessary to observe its own uncomplicated effects. It is important to point out that the experimental lesions in this group were very similar to those occurring when *Pasteurella* was found incidentally, apart from the virus changes in the bronchial epithelium.

All mice dead in 2 or 3 days, lungs intensely congested and injected, with no tendency to demarcation of lesions as in most of the other infections. Microscopically, in mice killed after 1 day, pronounced changes of collapse inflammation type, but lesions large, with abundant leucocytic exudate, so that collapse of alveoli was less complete than in other infections. After 2 and 3 days an intensification of this process, with confluence, the intervening alveoli containing more numerous polymorphs than in other infections, oedema of whole lung and congestion of inter alveolar capillaries marked, inflammation of vascular adventitia sometimes present but never prominent. Culture + (large numbers) in all cases.

### *H. pertussis*

Twenty two mice, 15 died in 2-4 days, 7 killed in 1-8 days.

Macroscopically, multifocal collapse in 1 day, considerable red patches (colour less intense than with virulent pneumococci) in 2 days, almost the whole lung sometimes involved in 3 days. Colour receding in 5 days, and subsequently lungs somewhat indurated, greyish pink, with a few pale spots where resolution most advanced.

Microscopically, initial collapse inflammation, centrifugal spread from primary foci in 2 days, with prominent perarteritis extending widely through the lungs, characterised by oedema with relatively scanty polymorphs, and comparatively little change in venous adventitia. Well marked diffuse oedema of whole lung and angioogenous pneumonia in many cases. In 3 days all these processes more conspicuous, pneumonia apparently developing simultaneously by centrifugal spread from primary foci and by invasion of alveoli from arterial adventitia, oedema fluid replaced in places by shreds of fibrin, moderate diffuse infiltration with macrophages containing altered blood pigment, degenerative changes in bronchial and tracheal epithelium in some cases, but accumulation of bacilli on the surface of bronchial epithelium not so conspicuous a phenomenon as Burnet and Timmins (1937) seem to have found it. In 4 days well established lobar pneumonia with exudate of fibrin, polymorphs, alveolar macrophages and other mononuclears. Subsequently progressive resolution, alveolar macrophages replacing other inflammatory cells. Culture lungs +, even at 8 days when resolution well advanced.

### Friedländer's bacillus (*Klebsiella pneumoniae*)

Two experiments (one with diluted 72-hour culture): 38 mice; 2 killed at 1 day, 2 survivors (diluted culture) killed at 8 and 11 days, remainder died in 2-6 days (mostly 2 or 3 days).

The most intense infection studied. Macroscopically, in 1 day multifocal collapse and petechiae, in 2-4 days extensive hæmorrhagic consolidation, in 8-day survivor one lobe greyish pink and indurated, in 11-day survivor subpleural petechiae only.

Microscopically, inflammation of vascular adventitia marked even in 1 day. Arteries, veins and to a less extent bronchi affected practically throughout lung, with great swelling, oedema, congestion, polymorphs and numerous bacilli; invasion from without of the other layers of the vessel walls especially in the region of the hilum, which appeared to be the primary point of invasion and from which inflammatory infiltration extended along the vessels. Focal collapse-inflammation relatively inconspicuous. In mice dying on 2nd or 3rd day periangitis still more marked and angiogenous pneumonia present, usually in several places; pleurisy; mediastinal lymphadenitis; tracheal and peritracheal lymphangitis. In those surviving 4-6 days, collapse inflammation more conspicuous, periangitis less widespread, no special intensity at hilum; in these less acute cases the process was developing by centrifugal spread from foci of collapse inflammation rather than along the vascular trees. In 8 days one collapsed lobe, infiltrated with macrophages; at 11 days patchy partial collapse with macrophages, traces of "epithelium" on alveolar walls. Culture + in lungs and blood of all except last two animals.

### Influenza virus A (W.S. strain)

Two experiments, one with a lightly centrifuged saline extract of infected mouse lungs (27 mice examined histologically), one with a bacteria-free filtrate (gradocol membrane A.P.D. 800  $m\mu$ ) of a similar extract (20 mice examined). Macroscopic appearances variable, but similar to those found with bacteria, especially *Pasteurella*.

Microscopically, in 6 mice killed after 1 day, bronchial epithelium somewhat swollen, vacuolated, cell margins blurred, with occasional slight desquamation or polymorph infiltration. In 2 days (2 dead, 4 killed) well marked epithelial degeneration, plugs of muco-pus in lumina, focal bronchiolar dilatation, peribronchitis. In 3 days (8 dead, 3 killed) all these processes more marked; denudation of bronchial wall in places. Progress of changes quicker with unfiltered virus. In 4-6 days degeneration still prominent, but in some cases evidence of regeneration of bronchial epithelium, at times with stratification. The foregoing changes are substantially the same as those described by Straub (1937, 1940), and are additional to anything seen in the bacterial infections. In the alveoli, on the other hand, though the findings are variable, they are of the same types as in bacterial infections. Lung cultures showed *Pasteurella* in many cases; other organisms included streptococci, staphylococci and *B. coli*; even in the second experiment (with filtrate) secondary bacterial invasion was found in 80 per cent. of animals. In those showing *Pasteurella*, the histological type of pneumonia was very similar to that obtained with *Pasteurella* cultures. Extensive bronchial epithelial changes without alveolar involvement were seen in a number of cases; in some of these the other lung had been sterile on culture.

### DISCUSSION

There is in existence a considerable literature on experimental pneumonia, especially of pneumococcal origin, but as this has been

comprehensively reviewed by Heffron (1939) we do not quote from it at length. It will suffice to indicate the bearing of previous work on some of the points in the present study.

In our experiments with bacteria it has always appeared that the infective material quickly reaches the alveoli and it is there that the first signs of inflammation are found. This is in agreement with the findings of Gaskell (1925), after intrabronchial injection of pneumococci in rabbits, that there is no damage to the bronchial epithelium or walls in the early stages. Permar (1923-24) found that experimental pneumonia in the rabbit originated as an acute inflammatory reaction of the trachea, bronchial tree, alveolar ducts, atria and alveoli, but that the severity of the reaction was greatest in the terminal bronchioles and the structures distal to them. Branch and Stillman (1924), after exposing mice intoxicated with alcohol to sprayed pneumococci, reported that the earliest lesion was an interstitial inflammation; inspection of their figs. 4 and 5 suggests that the lesion they so describe corresponds with our collapse inflammation. A similar difference in interpretation exists between us and Rake (1936), whose fig. 7 is stated to show "great thickening of the alveolar walls with the collection of leucocytes inside and outside the alveolar capillaries"; the picture itself shows a lesion which we should have called trabecular collapse inflammation, the alleged thickening of alveolar walls being in our opinion the result of close apposition of the walls. This condensation of lung tissue is clearly appreciated when the elastic tissue is stained.

Blake and Cecil (1920) injected pneumococci intratracheally in monkeys and believe that the first lesion is damage to small areas of the epithelium of large bronchi, which enables the pneumococci to penetrate to the peribronchial and perivascular interstitia in the region of the hilum. Thereafter the inflammatory process spreads along the vascular and bronchial adventitia and the septal tissue before organisms and exudate are found in the alveoli. After consolidation has developed the interstitial changes are less conspicuous. It will be seen that our view of the genesis of lobar pneumonia is essentially in agreement with that of Blake and Cecil, except that we have not found that the process necessarily originates at the hilum of the lung. This may well be a question of virulence, as our observations lend some support to the idea that in the most intense infections the process originated at and spread out from the hilum; moreover it was the usual finding in Friedländer infections. The presence of changes in the perivascular connective tissues has also been emphasised by Permar (1923-24), Gaskell (1925), Schöbl and Sellards (1926), Robertson, Coggeshall and Terrell (1933), and Rake (1936) for various animal species, though not all of them are prepared to accept the view that these changes constitute an important part of the mechanism in the genesis of pneumonia. Loosli (1942) believed interstitial involvement to be secondary and that the spread of

infection was from alveolus to alveolus, but his experiments were carried out on monkeys anaesthetised with morphine, which might result in considerable pulmonary oedema over the critical period. The importance of oedema in the spread of infection is also stressed by Gunn and Nungester (1936) and Hamburger and Robertson (1940), who gave intrabronchial injections of pneumococci to rats and morphinised dogs, accompanied by mucin and starch respectively. Loeschke's (1931) examination of human material led him to regard the oedema as essential to spread of the lesion. Coryllos and Birnbaum (1929) believe that the starting point of lobar pneumonia is a lobar collapse. We are inclined to believe that massive consolidation may result from either coalescence of small foci or rapid overwhelming invasion of the alveoli from a preliminary widespread interstitial infection, but are attracted by the theory that the classical lobar pneumonia of man may originate in the second of these ways. Amongst other things this would explain its fulminant onset, apparent uniformity in age throughout the lesion, absence of collapse in the consolidated alveoli and well marked associated pleurisy.

The primary change in influenza virus infections is degeneration and destruction of the bronchial epithelium, as previously described by Straub (1937, 1940). Thereafter the changes in the alveoli may be of a very diverse nature, ranging from extensive collapse to well developed pneumonia of various histological types. Bacteria are recovered from the pneumonic lungs in such a high proportion of cases that we are inclined to believe that the appearance of pulmonary consolidation is due to secondary bacterial invasion. The histological type of pneumonia probably depends upon the aetiological organism, though we can only claim to have proved this for *Pasteurella* infections. It therefore appears that the original statement by one of us (L. H.) that the pneumonia following influenza virus infections in mice is indistinguishable histologically from that caused by pathogenic bacteria, while in one sense valid, should be qualified by stating that the distinction can be made by an inspection of the bronchial epithelium.

#### SUMMARY

After intranasal administration of bacteria to anaesthetised mice, the earliest changes are found in scattered groups of alveoli, which show collapse and leucocytic infiltration. This occurs even with bacteria of low virulence. With virulent organisms, subsequent extension of pneumonia may take place either by centrifugal spread from the primary foci of collapse inflammation, or by extension along the vascular adventitia followed by widespread invasion thereof of the alveoli. The latter method appears to be the mechanism of production of lobar consolidation. There is no convincing evidence of the spread of infection up and down the bronchial lumina after the primary phase. In the most severe infections inflammation spreads

from the hilum. Changes in the bronchial epithelium are late, as opposed to influenza virus A infections, where they are primary and characteristic in type.

Our thanks are due to Mrs M. A. Smith for the coloured plate, to Professor H. B. Maitland for the strain of *H. pertussis*, and to the late Sir Patrick Laidlaw for the influenza virus.

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# FEATURES IN THE CONGO RED MOLECULE ASSOCIATED WITH THE INACTIVATION OF COMPLEMENT

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THE action of Congo red and a number of other dyes in inactivating complement was first shown by Klopstock (1924). He suggested that his results tended to support the view that complement inactivation is a physico-chemical phenomenon in which the colloidal state of the reacting substances is an important factor. The list of dyestuffs which he employed includes members of various chemical groups and they also vary considerably in respect of the physical states of their aqueous solutions.

Gordon (1930) also showed that Congo red and related dyes (Congo orange and Brilliant Congo G) inactivated complement as shown by tests on the bactericidal and hæmolytic activity of normal guinea-pig serum. This inactivation was unaccompanied by destruction of complement, since charcoal removed Congo red from an inactive mixture of complement-containing serum and dye, thereby restoring its hæmolytic activity. This reversibility, which was readily demonstrable, indicated an adsorption phenomenon of the dye on the complement. Similar inactivating effects were shown using Congo red against streptococcal hæmolysin, *Cl. welchii* hæmolysin (Gordon, 1931) and the anthracidal power of serum (Gordon and Wood, 1937). All these effects were readily reversible. Using the in-vitro anaphylactic technique, it was shown (Gordon and Robson, 1932) that Congo red caused inhibition of the anaphylactic reaction and that such inhibition was reversible.

## *The anti-complementary activity and substantivity of dyes of the Congo red series*

It seemed desirable to investigate whether this property of Congo red was in any way specific and how far the chemical structure of the dye could be modified without loss of activity. Since Congo red was the first synthetic direct cotton dye to be discovered (Böttiger, 1884), the first point seemed to be to decide whether substantivity (i.e. capacity to dye cellulosic fibres directly without a mordant) was related to anti-complementary action. For this purpose the two



isomeric disulphonic acids of Congo red, namely the bisazo dyes obtained from benzidine-2 : 2'-disulphonic acid and benzidine-3 : 3'-disulphonic acid respectively and two molecules of 1-naphthylamine-4-sulphonic acid were prepared and their action on complement determined. The former compound showed practically no affinity for cotton and the latter at least equal affinity to Congo red in comparative dye trials. Both dyes had even more anti-complementary effect than Congo red (table). The procedure was as follows. A 2 per cent. solution of each dyestuff in distilled water was freshly prepared and varying amounts from 0.05 c.c. upwards were added to 1.0 c.c. of normal guinea-pig serum. These mixtures were allowed to stand on the bench for 3 hours; 0.1 c.c. quantities of each were then added to 0.3 c.c. of 4 per cent. sensitised ox red cells, incubated at 37° C. for 1 hour and the degree of hæmolysis read.

These results suggested that the possession of substantivity by bisazo dyes of this series is in no way related to anti-complementary action. The mono-azo dye, benzene-azo-1-naphthylamine-4-sulphonic acid, was prepared and investigated for anti-complementary activity because it is a symmetrical fission product of Congo red and a compound of smaller molecular weight. It was found to have weaker anti-complementary activity—about half that of Congo red and less than half that of the Congo red disulphonic acids.

*The effect of replacement of amino groups by hydroxyl groups  
in Congo red dyes*

To investigate whether replacement of the amino groups in Congo red by hydroxyl groups had any influence on anti-complementary action, the dye Congo Corinth G (Berlin Aniline Co.), i.e. Congo red in which one amino group has been so replaced, was used. This dye was not sufficiently soluble for comparison by the technique usually adopted with Congo red, so that a 0.2 per cent. solution of the dye was tested against a 1 : 10 dilution in normal saline of normal guinea-pig serum. The results showed that Congo Corinth G had an anti-complementary activity parallel to that of Congo red. It was important to compare the activity of the dye obtained by replacing both amino groups of Congo red by hydroxyl groups, but as the bisazo compound obtained by coupling tetrazotised benzidine with two molecules of 1-naphthol-4-sulphonic acid was not sufficiently soluble, the corresponding bisazo dye from benzidine-2 : 2'-disulphonic acid was used. The results showed that this dye had marked anti-complementary activity comparable to that of Congo red disulphonic acid, thus demonstrating that replacement of the amino groups by hydroxyl groups in no way reduces the anti-complementary activity of dyes of the Congo red series. However, a comparison of the two mono-azo dyes—benzene-azo-1-naphthylamine-4-sulphonic acid (i.e. the symmetrical fission product of Congo red mentioned above) and

benzene-azo-1-naphthol-4 sulphonic acid (i.e. the symmetrical fission product of Congo red in which the amino group has been replaced by a hydroxyl group)—showed that the former was approximately twice

TABLE

*The effect of various dyes on complement activity*

Mixtures of guinea pig serum and dyes		Effect of adding 0.1 c.c. of the mixture to 0.3 c.c. sensitised ox red cells
Guinea pig serum	Dye	
1 c.c. undiluted	0.05 c.c. } 0.1 " } Congo red (2 per cent ) 0.15 " } 0.2 " }	{ 4 4 0 0
	0.05 " } Benzidine 2, 2' disulphonic acid bisazo dye 0.1 " } (2 per cent ) 0.15 " }	{ 4 0 0
	0.05 " } Benzidine 3, 3' disulphonic acid bisazo dye 0.1 " } (2 per cent ) 0.15 " }	{ 4 0 0
	0.05 " } 0.1 " } Benzene azo 1 naphthylamine 4 sulphonic 0.15 " } acid (2 per cent ) 0.2 " } 0.25 " }	{ 4 4 4 4 0
	0.05 " } Bisazo dye from benzidine 2, 2' disulphonic 0.1 " } acid and 1 naphthol 4 sulphonic acid 0.15 " } (2 per cent )	{ 4 0 0
		Effect of adding 1.0 c.c. of the mixture to 0.3 c.c. sensitised ox red cells
1 c.c. diluted 1/10	0.05 c.c. } 0.1 " } Congo Corinth G (0.2 per cent ) 0.15 " }	{ 4 4 0
	0.1 " } 0.2 " } Acid fuchsin (2 per cent ) 0.3 " }	{ 4 2 0
	0.05 " } 0.1 " } Basic fuchsin (0.2 per cent ) 0.2 " } 0.3 " }	{ 4 4 2 P
	0.05 " } 0.1 " } Basic fuchsin (0.5 per cent )	{ 4 0

4 = complete hæmolysis

2 = partial hæmolysis

0 = no hæmolysis

P = precipitation

as anti-complementary as the latter. The comparison had to be carried out using, in the case of the latter dye, 0.2 per cent. solutions and 1/10 dilutions of guinea-pig serum in normal saline, because

of its lower solubility. Thus in contrast to the results with dyes of the Congo red series it appears that replacement of the amino group by a hydroxyl group in the simpler mono-azo dye has a considerable influence on anti-complementary activity.

*The anti-complementary action of other chemical substances*

In view of the lack of definite evidence of any specificity attached to the Congo red molecule, and also because of Klopstock's previously mentioned results of anti-complementary action shown by widely differing chemical groups of dyes, other substances were examined. For this purpose we selected eosin G (a phthalein dye), basic fuchsin and acid fuchsin (triphenylmethane dyes). We found that eosin was equal to Congo red in its anti-complementary activity but, on the other hand, there was a striking difference in the behaviour of basic and acid fuchsin. Owing to the low solubility of basic fuchsin it was found necessary to use 0.5 and 0.2 per cent. solutions in place of the more usual 2 per cent. solutions. The results (table) show that basic fuchsin is approximately ten times as anti-complementary as acid fuchsin. It is interesting that the introduction of sulphonic acid groups should so greatly reduce the anti-complementary activity of basic fuchsin.

In this connection it is of interest to record that Klopstock (1932), from investigations of the action of heparin and Germanin (Bayer 205) on immune reactions, suggested the possibility that substances which change the iso-electric point of proteins towards the acid side exercise an increased influence on complement. It appears possible that the differing anti-complementary activities of acid and basic fuchsin may be of this nature. Schmidt (1925-26) and Klopstock (1932), among others, showed that Germanin would inactivate complement and this observation we have confirmed by using Antrypol (I.C.I.), which is the English equivalent of Germanin. The initial interest in this compound was partly due to the fact that although it is a colourless substance it possesses marked substantivity. We have found it to be slightly more anti-complementary than Congo red; of a 2 per cent. solution 0.1 c.c. inactivated while 0.05 c.c. failed to inactivate the complement of 1.0 c.c. of normal guinea-pig serum.

The anti-complementary action of Congo red was not inhibited when dilute solutions of the dye were mixed with diluted serum. This effect was shown in the following mixtures: undiluted serum and 2 per cent. Congo red solution; serum diluted 1:4 with normal saline and 0.5 per cent. Congo red solution; serum diluted 1:10 with normal saline and 0.2 per cent. Congo red solution.

*Reversibility effects*

It has been shown previously (Gordon, 1930) that the inactivation of complement by Congo red can be prevented by adsorbing the dye on charcoal. In the case of the inactivation of streptococcal hæmolyisin by Congo red, Gordon (1931) showed that this action was reversed by adding cuprammonium artificial silk to the mixture, allowing it to stay in contact for a short time and expressing the fluid, which was then again hæmolytic. This method was now used in the case of certain of the dyes already mentioned, employing either artificial silk or natural silk as the reversing agent. Thus the anti-complementary activity of benzene-azo-1-naphthylamine-4-sulphonic acid was reversible with cuprammonium artificial silk but more so with natural silk. Congo red inactivation was reversible with natural silk, but was more easily reversible with cuprammonium artificial silk. The inactivation of complement by Antrypol was not reversible with either. In the case of the disulphonic acids of Congo red, the complement inactivation by the bisazo dye from benzidine-2:2'-disulphonic acid (not substantive) was reversible with the artificial but more so with natural silk, whilst the bisazo dye from benzidine-3:3'-disulphonic acid was reversible with both agents about equally.

*Discussion*

The point to be noticed in these experimental results is that the inactivation of complement in normal guinea-pig serum by dyestuffs and related compounds is not associated with any important chemical changes in the constitution of the compounds, but rather consists in a simple adsorption of the dye on complement or at most in some loose chemical combination. The fact that dyes of widely differing chemical constitution can inhibit the action of complement and that agents such as cotton, silk and even charcoal can reverse this action in the case of suitable dyes supports this conclusion. There is a lack of any relationship between substantivity and the anti-complementary activity of dyes of the Congo red series. Modifications of the chemical structure of Congo red such as the introduction of further sulphonic acid groups or replacement of the amino groups by hydroxyl groups have little or no influence on anti-complementary activity. However, in the case of the symmetrical fission product of Congo red—benzene-azo-1-naphthylamine-4-sulphonic acid, the molecular weight of which is approximately half that of Congo red. When the amino group of benzene-azo-1-naphthylamine-4-sulphonic acid is replaced by a hydroxyl group, the anti-complementary activity is still further reduced. There was a marked contrast in the anti-complementary activities of basic fuchsin and acid fuchsin.

*Summary*

The anti-complementary activity of dyes of the Congo red series appears to be dependent more on the molecular size of the reacting substance than on any specific grouping within the molecule.

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# THE EFFECT OF AGAR DEPTH IN THE PLATE METHOD FOR THE ASSAY OF PENICILLIN

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Of the methods at present available for the assay of penicillin the plate technique is considered the most suitable for all general purposes since, unlike the dilution and turbidimetric methods, it requires no special equipment or medium which is not readily available to any laboratory undertaking routine bacteriological work and involves the preparation of but one accurate dilution of the solution under test. Many modifications of the method have been described (Foster and Woodruff, 1944; Schmidt and Moyer, 1944; Heatley, 1944) and a brief but excellent review of the various techniques used is given by Garrod and Heatley (1944-45). When current techniques are employed for assays demanding a reasonable degree of accuracy, a considerable number of plates must be used for each estimation. Thus, the error of plate assays put up in quadruplicate by experienced workers is of the order of  $\pm 15$ -20 per cent. It has been shown that, in order to obtain an accuracy of  $\pm 7.5$  per cent. by the Oxford method, sixteen plates must be used for each assay, while eight plates are probably necessary if an accuracy within 10-15 per cent. of the true value is desired (Major S. T. Cowan, R.A.M.C., 2nd Interim Report to the Director of Pathology, War Office (restricted), September 1944). In unskilled hands the error is likely to be appreciably greater.

The object of the work here described was to investigate the plate assay method with a view to finding and controlling the factors causing variability.

## *General details of technique used*

*Test organism.* Staphylococcus CN876 (source unknown) was used throughout the investigation. This strain showed a tendency to form a deposit when grown in standard nutrient broth but this was not found to interfere with the results. Subculture was performed at intervals of not more than four days.

*Media.* (1) *Standard nutrient broth* was prepared according to the following formula.

"Lemco" meat extract . . .	10 g.
Peptone (Evans) . . .	10 "
Sodium chloride . . .	5 "
Water to . . .	1000 ml.

After autoclaving and filtration the pH was adjusted to 6.8. The broth was dispensed in 25 ml. volumes into neutral glass, screw-capped bottles and re-

sterilised by autoclaving. This stock was decanted as required in 5 ml. volumes into test-tubes and inoculated with a 2 mm. loopful of a 24-48-hour broth culture of staphylococcus in the same medium. Standard nutrient broth constantly yielded a viable count of 100-150 million organisms per ml. after 18-24 hours at 37° C.

(2) *Standard nutrient agar*. The basis of this medium was standard nutrient broth with 2.5 per cent. agar fibre added, buffered with one-tenth its volume of *M*/15 phosphate buffer at pH 6.8. The medium was prepared in bulk and dispensed in suitable volumes in neutral glass screw-capped bottles.

*Standard penicillin solutions*. Sub-standard barium salt was only available during the initial stages of this work. Consequently a stock standard solution of 10,000 units per ml., prepared from a commercial (Lederle) sodium salt and standardised against the sub-standard by a dilution method in tubes, was used. From this stock working standards were made every 4-6 weeks. All dilutions of penicillin were prepared in distilled water buffered at pH 6.8 and preserved with chloroform at 4° C. in neutral glass screw-capped containers.

*Cylinders*. These were of glazed porcelain, 9 mm. high  $\times$  7.5 mm. external diameter and bevelled from within outwards at one end. After use they were cleaned by washing, followed by boiling for from 5-10 minutes in 30 per cent. nitric acid and further washing in running water. Sterilisation was effected by dry heat at 140° C.

*Method of seeding*. A pre-seeding technique was adopted, since surface seeding during the hot weather in the tropics was found to produce too high a contamination rate. It has been found that the most clearly defined zones of inhibition are obtained when the concentration of staphylococci is approximately 250,000 per ml. agar. With concentrations much higher than this the zone edges tend to become diffuse while with lower densities the colonies are too discrete and accurate measurement of the zones becomes difficult. Once the correct dilution for any particular batch of broth has been found, this dilution may be used for the rest of the batch without further counts provided the broth is stored in neutral glass bottles.

### *Experimental findings*

There are many intrinsic sources of error in the plate method which are readily controlled. Such factors are concentration of agar and its pH, density of the inoculum, the manner of sealing the cylinders to form a fluid-tight joint with the agar, and the conditions of time and temperature observed between the addition of penicillin to the cylinders and incubation. Roughly the same volumes of agar should be used in plates of similar size. The effects of these factors were worked out and the results were found to conform to the findings of others. However, after the conditions of assay had been standardised to eliminate these sources of error, approximately the same degree of variation between assay values occurred as had been noted elsewhere.

Attention was drawn to the effect of small variations in agar depth by the observation that oval instead of circular zones of inhibition occurred on a plate whose bottom was slightly convex inwards, the long axis of the ovals being extended towards the centre of the plate where the agar was thinnest. It was decided to investigate in detail the effects of variation in depth of agar on the assay values given by various concentrations of penicillin.

Plates 88 mm in diameter, with smooth flat floors of thick glass were used. Agar was melted, seeded in bulk and carefully measured volumes pipetted into plates in duplicate so as to give a series of agar depths ranging from 0.5 to 10 mm. Precautions were taken to ensure that the plates were level during pouring. Penicillin in concentrations of 4.0, 3.0, 2.0, 1.0, 0.5 and 0.25 units per ml was pipetted into cylinders arranged peripherally on each plate. After 45 minutes at 4° C the plates were incubated. Assay values were measured after 18 hours at 37° C. Results are shown in fig 1, in which variations in assay value for different concentrations of penicillin are plotted against agar depth. This experiment was also carried out in quadruplicate using a different batch of agar, with similar results.

It will be seen that for each concentration of penicillin, once the agar falls below a certain depth, which is greater the higher the concentration, the curve which had previously been parallel to the abscissa begins to rise and the more shallow the agar the steeper this rise becomes. Moreover, as the concentration of penicillin decreases, the upward sweep of the curve from the horizontal becomes increasingly abrupt and steep. The behaviour of these curves may be explained if it is supposed that penicillin diffuses through the agar roughly in the form of a hemisphere. When the depth of agar is greater than the radius of significant diffusion for any given penicillin concentration diffusion will occur freely and no alteration in the surface zone of inhibition will be expected for any additional increase in depth. It has been demonstrated that the assay values of 3.0, 2.0 and 1.0 units of penicillin per ml do not alter when 40 mm depth of agar is used instead of 8 or 10 mm. If, however, the agar depth becomes less than the radius of maximum significant diffusion, free diffusion downwards will be impeded and the penicillin displaced outwards, thus increasing the assay value. Moreover, for any given change in agar depth we would expect a proportionately greater exaggeration of the small zones produced by the lower penicillin concentrations than of the zones resulting from higher concentrations, once the bottom of the plate has begun to impinge on the sphere of effective diffusion. Reference to fig 2 will make this clear. The diffusion of three strengths of penicillin is represented "A" diffuses to a depth of 2 mm, "B" to 5 mm and "C" to 10 mm. A decrease of 1 mm in a depth of agar just equal to the radius of diffusion would displace rather less than one half of the penicillin diffusing from "A", less than one fifth of that from "B" and less than one tenth of that from "C". This example presents a rather gross simplification of what must actually happen, since the concentration of penicillin is rapidly diminishing as it diffuses away from the cylinder, a fact that would tend to compensate the effects described. Again it will be seen that with certain depths of agar the assay value of higher concentrations may be exaggerated while those of lower



concentrations remain unaffected. In fig. 2 if the depth of agar lay between 5 and 10 mm. the assay value of "C" would be exaggerated while those of "A" and "B" would not.

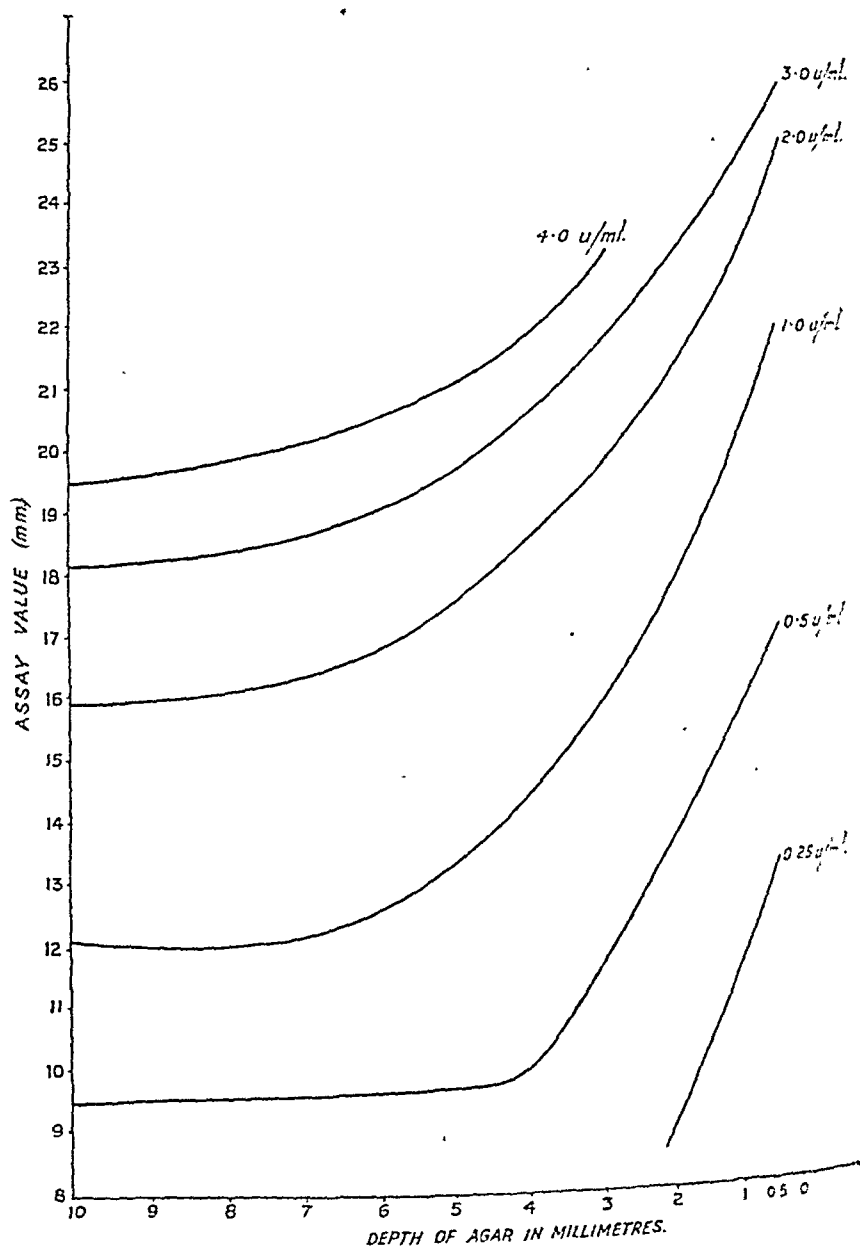


FIG. 1.—Effect of depth of agar on penicillin assay value.

The plate assay values of penicillin, therefore, may be regarded as remaining constant until the depth of agar becomes less than the significant radius of diffusion, but a decreasing depth below this

limit exaggerates the true value more rapidly as the concentration of penicillin diminishes. Examination of fig. 1 shows that for any given penicillin concentration the assay value begins to increase at agar depths considerably in excess of the demonstrable radius of diffusion as given by the formula

$$\frac{\text{Assay value} - \text{diameter of cylinder}}{2}$$

It must be remembered, however, that penicillin continues to diffuse beyond the point at which it just inhibits the growth of staphylococci.

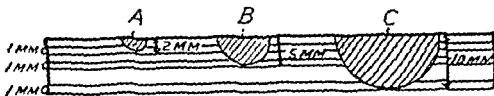


FIG. 2.—Diffusion of three strengths of penicillin in 10 mm. depth of agar.

Two important principles emerge from these considerations. The first is that the depth of agar used in the plate assay method is a very important factor in determining results: the second, that the true relationship between penicillin concentration and assay value can only be expressed by curves prepared from assays performed on deep agar.

*The probable error introduced by small differences in the depth of agar in assay plates*

This error can be gauged from any curve in fig. 1, which shows that, for agar depths greater than 7.8 mm., variations in depth do not affect assay values significantly for penicillin concentrations of 2.0 units per ml. or less. Much smaller depths have been used by other workers. The Oxford method (Heatley, 1944) gives a depth of approximately 2.7 mm., Schmidt and Moyer's method (1944) approximately 3.2 mm., Foster and Woodruff's method (1944) approximately 1.7 mm. Fig. 1 shows that at these agar depths small variations result in big changes in assay value. Thus a variation in depth of 0.25 mm. in a plate of 2.0 mm. mean depth gives the following approximate variations in assay value: 0.65 mm. for 2.0 units, 1.0 mm. for one unit and 1.2 mm. for 0.5 units per ml. The significance of these variations may be assessed from fig. 3, drawn from the same data as fig. 1 but relating assay value to penicillin concentration. For the curve corresponding to an agar depth of 2.0 mm. these assay value variations give a variation in the estimation of penicillin concentration of approximately 24 per cent., 20 per cent. and 12 per cent. for 2.0, 1.0 and 0.5 units per ml. respectively. The decrease in final error for decreasing strengths of penicillin, in spite of the greater absolute

variation in assay value as the penicillin concentration becomes less, is due to the over-compensation produced by the progressive steepness of the curves in fig. 3 for lower concentrations.

A variation of 0.5 mm. in the depth of agar on a plate is impossible to appreciate with the naked eye. There are three ways in which such variation may occur. (1) Deviation of the floor of the plate from the horizontal during pouring. The calculated deviation required to produce a 0.5 mm. variation in a 100 mm. plate is rather less than

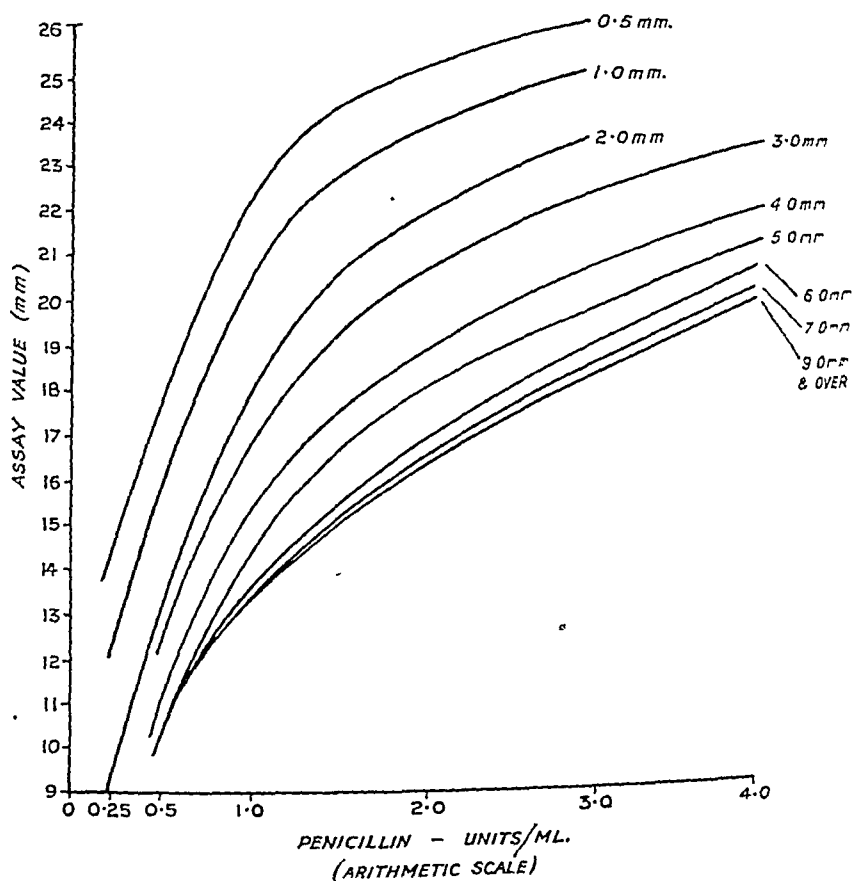


FIG. 3.—Relation of assay value to penicillin concentration for varying depths of agar.

0.3°. (2) By slight irregularities in or divergence from a plane surface of the floor of the plate. (3) By slight displacement upwards of agar within the cylinders as these bed down a little after heating.

It seems probable that small and unnoticed variations in agar depth form the greatest intrinsic cause of error in the plate assay method. Two methods of overcoming this variation are:—(1) The use of plates with flat, plate-glass floors, together with some delicate levelling device to maintain the plates on a horizontal plane during pouring. (2) The use of agar in depths of about 8-10 mm. This

method is the simpler and more practical of the two and was found to have an additional advantage over shallow agar in that the surface growth of pre-seeded staphylococci was much more luxuriant and the clarity of inhibition zones correspondingly enhanced.

*Recommended procedure for plate assay*

Standard nutrient agar is melted and poured into the required number of plates to give a depth in each plate of about 8 mm.; 88 mm. plates require about 50 ml., 100 mm. plates about 65 ml. of agar. Pouring can best be done with a 100 ml. measuring cylinder. The plates are allowed to set. One to 1½ hours before the time of assay the plates are placed on a board fitted with wooden runners separated by a width slightly greater than that of the plates and of such a height that the lids are lifted about ¼ inch above the level of the plate walls while still allowing considerable overlap between the sides of the lids and plates (fig. 4). The plates are placed in the incubator at 37° C. For every assay plate to be put up, 4-5 ml. of standard agar are now melted, cooled to 45-48° C. and seeded with the correct dilution in saline of staphylococcus broth culture. The pre-seeded agar is well mixed by shaking and left in the water-bath at 45° C. for a few minutes to allow bubbles to rise to the surface. The plates are now taken out of the incubator two at a time. About 3 ml. of pre-seeded agar is pipetted on to each, spread quickly over the surface by

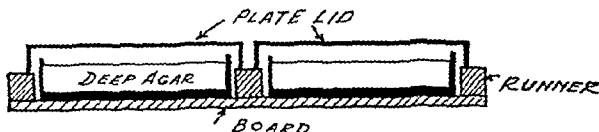


FIG. 4.—Method of supporting lids of deep agar plates.

rolling and then run off into disinfectant by holding the plate upside down at an angle, followed by a few brisk shakes, when the agar begins to fall in drops. The lid is put on and the plate quickly returned to the bench to set. The plates are then placed over a template, the lids propped up and the cylinders, after heating slightly by passing two or three times through a bunsen flame, dropped on the surface over the points marked on the template. The degree of heating should preferably be such that only the faintest hiss is heard when the cylinder makes contact with the agar. Results have shown that careful standardisation in the heating of the cylinders may be ignored with deep agar, although still an important factor when levelled shallow agar plates are used. As each plate is completed it is returned to the board and the lid placed over it, resting on the runners.

The next stage is the addition of standard and unknown penicillin dilutions to the cylinders. This is done with a sterile Pasteur pipette which is rinsed in boiling water between the addition of one dilution and the next. It is desirable to fill the cylinders to the top. If less than about 5 drops of penicillin are added the size of the assay value decreases. Plates are then transferred to the refrigerator for 45 minutes (or any other standard time) and thereafter to the incubator. The following morning the zone diameters are measured with a pair of sharp-pointed dividers and the assay values read off on a millimeter scale.

The following should be noted.

(1) The test organism most widely used is *Staphylococcus* no. 6571 of the National Collection of Type Cultures (England). Foster and Woodruff favour

a sensitive strain of *B. subtilis* and, especially when a pre-seeding technique is adopted, a spore suspension offers great advantages in the ease with which the inoculum can be standardised. Very clean-cut zone margins is another advantage claimed. It has recently been shown that penicillin consists of three active fractions termed penicillin I, II and III (F, G and X in American nomenclature). The greater proportion of the activity of modern commercial preparations is due to penicillin II, while penicillin III is not present in significant amounts. Since *Staphylococcus* 6571 and its American counterpart are the only organisms known to be equally sensitive to penicillins I and II and since the object of assay is the assessment of therapeutic efficacy it has been recommended that, in the present state of our knowledge, these staphylococci should be the organisms of choice for assay purposes. The International Penicillin Standard now adopted is prepared from penicillin II, the International Unit being 0.6 microgram of the pure sodium salt (1666 units per mg.). (See "Report on the International Conference on a Standard and Unit for Penicillin, October, 1944", held under the auspices of the League of Nations Health Organisation.)

(2) The seeding technique recommended combines the advantages of pre-seeded agar with the clarity of zone definition given by surface seeding with broth culture, but has not been found to produce any variation in assay value such as Heatley (1944) described as occasionally occurring in the quadrant from which excess broth culture is withdrawn in his method. The depth of pre-seeded agar given by the method described is just over 0.1 mm. Pre-seeded agar in greater depths (0.5 mm. or over) has been found frequently to produce rather diffuse zones with the higher concentrations of penicillin used and sometimes, with lower concentrations, two clear-cut zones of inhibition, the outer on the agar surface and the inner just below the surface, where staphylococcal growth still yields a definite turbidity.

(3) The use of the board (fig. 4) is threefold. Firstly, it prevents water condensation on the plate lids; secondly, it raises the lids sufficiently to allow head-room for the cylinders resting on the deep agar when plates of common pattern less than 20 mm. deep are used; thirdly, by allowing evaporation, the tendency towards the formation of surface moisture by the "sweating" of deep agar is counteracted, with the result that a luxuriant but dry surface growth is obtained after incubation.

(4) The usual concentrations of standard penicillin employed are 2.0, 1.0 and 0.5 units per ml. With deep (2.5 per cent.) agar the assay value of 0.5 unit per ml. is very small and difficult to measure without removing the cylinder from the plate: 0.75 unit per ml., however, gives an easily measurable zone. A standard range of 0.75, 1.0 and 1.5 units per ml. or 0.75, 1.5 and 3.0 units per ml. might constitute a more suitable series. Alternatively, bigger zones would be given by refrigeration for a longer time prior to incubation.

(5) Two alternative methods of sealing the cylinders have been tried. In one, 1.5 per cent. pre-seeded agar was used and the cylinders simply placed on its surface. Considerable variation and distortion of zones occurred owing to leakage. In the other method the cylinders were laid on the agar surface and fixed by the addition to them with a standard pipette of three drops of molten agar. Approximately double the concentrations of penicillin had to be used. The amount of variation in assay value between plates was less than that given by heat fixation when shallow agar was used but greater in the case of deep agar.

#### *Comparison of results of deep and shallow plate methods*

A series of 50 consecutive assays was carried out by both methods, not more than 10 assays by each method being put up on any one

day. The deep agar technique was that described above, 88 mm. plates with flat plate-glass floors being used. For the shallow method, 12 ml. of pre-seeded agar were carefully measured and pipetted into similar plates, giving a depth of 2 mm. Both series of plates were poured on an approximately level glass-topped table. All other stages of assay were identical. Five concentrations of penicillin, 2.0, 1.25, 1.0, 0.75 and 0.5 units per ml., were employed. For purposes of statistical assessment of error the 1.25 and 0.75 unit per ml. values were regarded as "unknowns".

For the statistical analysis of the results obtained I am greatly indebted to Major D. A. K. Black, R.A.M.C., and Mr C. K. Dilwali. Assessment of error for deep and shallow agar methods was worked out for assays in duplicate. The data from which the limits of accuracy were worked out were the penicillin concentrations estimated by interpolating the assay values of the 1.25 and 0.75 concentrations of penicillin on the curve of the 2.0, 1.0 and 0.5 units per ml. concentrations for plates in duplicate. The results of statistical analysis are given in the table.

TABLE  
*Statistical analysis of assay results*

1. Variability of assay values for penicillin concentrations of 2.0, 1.0 and 0.5 units per ml				
	Mean (mm)	Range (mm)	Standard deviation (mm)	Coefficient of variation (per cent)
<b>Shallow agar (50 values)</b>				
2.0 units/ml.	21.0	19.5-22.0	$\pm 0.56$	2.7
1.0 " "	17.9	16.0-19.75	$\pm 0.68$	3.7
0.5 " "	12.8	11.5-14.0	$\pm 0.59$	4.6
<b>Deep agar (50 values)</b>				
2.0 units/ml.	15.6	15.0-16.5	$\pm 0.35$	2.2
1.0 " "	11.6	10.5-12.75	$\pm 0.39$	3.4
0.5 " "	8.4	8.0-9.0	$\pm 0.35$	4.2
2. Error of results for assays carried out in duplicate				
Standard deviation (units)	Mean (units)	Coefficient of variation	Limits of accuracy (per cent.) (P = 0.05)	
<b>Oxford method</b> (Garrod and Heatley, 1944-45)		0.0 for quadruplicate assays	$\pm 24.9$	
<b>Shallow agar (levelled plates)</b>				
0.75 units/ml $\pm 0.081$	0.77	*10.5	$\pm 21.17$	
1.25 " " $\pm 0.091$	1.20	*7.6	$\pm 14.26$	
<b>Deep agar</b>				
0.75 units/ml $\pm 0.035$	0.78	*4.5	$\pm 9.16$	
1.25 " " $\pm 0.057$	1.29	*4.44	$\pm 8.94$	

\* The coefficient of variation values for deep and shallow agar methods quoted above are calculated from the theoretical mean of 0.75 and 1.25 units/ml. respectively and not from the actual mean.

The revised figures for the limits of accuracy of the Oxford method are  $\pm 17.6$  and  $\pm 24.9$  per cent. for assays in quadruplicate and duplicate respectively (Garrod and Heatley, 1944-45). For the same value of  $P(=0.05)$  the accuracy of assays carried out in duplicate by the deep agar method is  $\pm 9.15$  per cent. for 0.75 unit per ml. and  $\pm 8.94$  per cent. for 1.25 units per ml. That for duplicate assays by a shallow agar method using special plates and a reasonably level table for pouring is  $\pm 21.17$  per cent. for 0.75 unit per ml. and  $\pm 14.26$  per cent. for 1.25 units per ml. concentration of penicillin.

### *Summary and conclusions*

1. The relationship between depth of agar and assay value for various concentrations of penicillin in the plate assay method is demonstrated.

2. Very small variations in agar depth are shown to exercise a marked effect on the assay value when the mean depth of agar is small, but this effect disappears when deep agar is used.

3. A method of assay employing deep agar plates is described in detail.

4. The accuracy of this method is shown to be markedly superior to that of other methods hitherto described.

My thanks are due to Capt. Leela Lai, I.A.M.C., for assistance in this work, to Lt.-Col. R. N. Phease, R.A.M.C., for advice and criticism and to the D.M.S., India, for permission to publish this paper.

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## AN EXPERIMENTAL APPROACH TO THE PROBLEM OF TRAUMA AND TUMOURS

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THE relationship, whether causal or fortuitous, of trauma to tumours in human pathology remains debateable (Ewing, 1935, Schmid, 1936 37, Behan, 1939, Berenblum, 1944) It can sometimes be established with certainty that simple injury in the form of a surface blow, burn or penetrating wound preceded the growth of a tumour at a particular site The problem then resolves itself into a discussion whether the injury caused the tumour or whether the association was due to chance Certain criteria were suggested by Ewing and by Schmid in debating this problem All common experience and experiments—for example those of Marsh (1917)—in which injuries of various kinds operate alone without causing tumours make it certain that simple uncomplicated trauma does not cause cancer Injections of non specific chemical substances, on the other hand, are often effective, for example light green into rat subcutaneous tissues (Schuller, 1937), hydrochloric acid in acid potassium phthalate in mice (Suntzeff *et al*, 1940) and zinc chloride into the testicles of adult roosters (Michalowsky, 1928, 1929 30, Bagg, 1936) More recent work suggests that another approach to the problem might be made by assuming that other factors also play a part In the present paper the combined action of simple injury and of a known carcinogenic agent is considered and put to the test The assumption of the intervention of a hypothetical carcinogenic agent prior to injury has this justification Cancer producing agents are known to exist outside the body (for example tar, shale oil and radium) and it is not improbable that others are made within the body itself The conversion of the natural substances desoxycholic acid and cholic acid into methyleholanthrene by simple chemical transformation provided Cook *et al* (1937) with grounds for thinking that similar changes might occur *in vivo* Search in various tissues and body fluids for naturally occurring carcinogenic compounds led to the extraction from the liver of a product which caused sarcomas on injection into mice (Berman, 1935 36, des Ligneris, 1936, Schabad, 1937, Hieger, 1940) That identical or similar substances are free to act in life has not yet been proved, but the possibility exists and the assumption



of action by some undetected exogenous agent seems not to be outside the realm of probability.

It is known that wound healing combined with the application of tar may localise tumours at the site of healing in mice (Deelman, 1923-24; Pullinger, 1943, using synthetic chemical carcinogens); the combined action may even increase the number of tumours three-fold at the wound site beyond those that grow elsewhere in the treated skin of rabbits (MacKenzie and Rous, 1941). This experimental association again affords a basis for suggesting that those human tumours which are preceded at a particular site by injury are in part due to the action of some undetected extraneous or endogenous carcinogenic agent. The injury might then act, as MacKenzie and Rous have suggested, by stimulating latent neoplastic change. Yet even if these assumptions are proved true—that a carcinogenic agent may in fact intervene and the cells so affected lie latent until aroused to proliferation by added injury—the human problem is not therefore necessarily solved. In almost all the experiments using combined lesions every animal developed tumours at sites other than the one subjected to the action of both agents. The human problem is not one of site but of incidence. The information required is whether the individual's chances of developing even a single tumour are likely to be increased by simple injury above those due to the hypothetical cancer-producing agent acting alone. That this is the particular human problem may be illustrated by reference to occupational pre-cancerous conditions such as X-ray dermatitis. Efforts are always made to protect the affected part, yet it is not doubted that tumours may arise there quite apart from any added injury. What is unknown is the measure of the added risk of a single unrepeatable injury.

In certain experiments in which agents other than mechanical trauma were combined with synthetic carcinogenic compounds, a real increase in incidence among the animals at risk appears to have been found. Thus Rondoni and Corbellini (1936) raised the incidence of skin tumours in mice by treatment with 1:2:7:8-dibenzacridin combined with three successive burns at the same spot. Three out of seven mice bore localised tumours. Two more tumours probably occurred on burns, though this is not exactly stated. Among the control unburnt animals there was one doubtful tumour only. Thus the incidence was raised.

Tumour incidence was apparently raised by the combined action of a very dilute solution (0.05 per cent.) of benzpyrene in acetone used once weekly and croton oil resin dissolved in acetone (Berenblum, 1941). The number of tumour-bearing mice increased from a maximum of 6.5 per cent. without croton resin to 80 per cent. with it, in an observation period of 20-22 weeks.

In an experiment recorded later (exp. IV; fig.) it was found that if the mice were kept alive beyond this time, 38 out of 39 treated with benzpyrene alone did eventually develop tumours. There was, it is true, an added stimulus—an act of scarification and an excision—but in 25 out of these mice there were no localised tumours at either site. Thus the scarification and wounding did not stimulate these 25. Of the 13 tumours which appeared on wounded areas, only five arose there first before others were seen elsewhere.

Thus 33 out of 38 first tumours (86 per cent) cannot be ascribed to anything but the very dilute solution of benzpyrene

The effect of the croton resin in Berenblum's experiments was to reduce the latent period, but owing to the limited period of observation it is less certain that it increased tumour incidence. Similarly Kline and Rusch (1944) allowed only a 24 weeks' observation period in a comparison of skin painting of mice with methylcholanthrene alone for 16 weeks and methylcholanthrene for the same period followed by croton oil resin. The results as stated prove only that croton resin hastened the tumour incidence. They do not prove that total incidence was increased though it may have been.

Expt IV (fig) of the present paper suggests that when highly active carcinogenic chemicals are used in doses below the optimum for tumour production by those substances, a dosage range may be reached which merely delays the appearance of tumours without reducing the total incidence. This possibility needs to be considered when judging the effect of the combined action of several agents. Better proof of an actual increase in incidence as well as a reduction in the latent period was provided by Mottram (1944). He obtained no tumours in six mice treated successively with croton oil and a single application of benzpyrene (0.02 c.c. of a 0.03 per cent solution), six tumours in five out of six mice treated successively with one application of benzpyrene and then with croton oil for 20 weeks, and 25 tumours in six out of six mice treated in succession with croton oil, benzpyrene (one application only), and then croton oil for 20 weeks. A single application of benzpyrene is ineffective in producing tumours, so that the combined treatment must have been responsible for this high incidence.

Another striking example of the ability to cause tumours by the combined action of two agents, neither of which is effective alone in so low a dosage, is provided by the work of Lacassagne and Vincent (1929), Lacassagne (1933), Burrows *et al* (1937) and Burrows and Clarkson (1943). These authors produced foci of inflammation in the subcutaneous tissues of rabbits by injections of streptococci, diatomaceous earth or powdered silica and exposed them to single doses of 600 r of X rays. A large proportion of all the rabbits developed sarcomas at the sites of injection. Burrows and Clarkson failed to produce tumours with this dose of radiation alone and found that, in the combinations of agents and doses used, 600 r was the minimum effective dose.

The experiments now to be recorded were undertaken to determine whether a single act of mechanical injury in the form of a skin excision had the power to increase the effectiveness of certain carcinogenic chemical compounds as measured by tumour incidence. In comparison with the two previous examples of combined action, namely benzpyrene and croton oil, and X rays and silica, wound healing had little stimulating action.

Before describing these experiments it is necessary to recall the observations of Orr (1938, 1940) of widespread and focal alterations in the dermis consisting in the formation of fine fibred non refractile collagen, fibrosis and scars, and also of focal gaps in elastic tissue after painting mouse skin with carcinogenic chemicals. The more potent the agent in producing tumours the more rapidly do all these alterations occur. Orr claimed no peculiar relationship between tumour formation and these widespread changes, but he considers that focal fibrosis or scars and defects of elastic tissue have specific importance. If these changes are in fact invariably present as the

result of the application of specific chemical substances, then it may be argued that, whether one adds a mechanical injury of set purpose or not, some kind of destructive inflammatory lesion in the dermis does always precede tumour growth.

To test the constancy of this occurrence, very young papillomas which had been produced with 2-methyl-3:4-benzphenanthrene dissolved in acetone were removed, together with surrounding skin, as soon as they were noticed and after killing the mice. The tissues were fixed in absolute alcohol and sections stained by Weigert's method for elastic fibres and by Weigert's iron hæmatoxylin and van Gieson's stain. This carcinogenic chemical has an average latent period of 155 days and yields tumours in 75 per cent. of mice (Iball, 1939). Little gross damage was caused during application. Sixteen of the papillomas which appeared were suitable for examination of the dermis because they had not yet become invasive. In ten no defect or gap in the elastic fibres was seen; in six there were either gaps or defects consisting of isolated short lengths of fibres or little masses of curled fibres. All these papillomas had dilated blood vessels at their bases. There was slight proliferation of connective tissue cells and fibres supporting the vessels of supply. Nothing more than could be accounted for in this way and no evidences of former destruction were seen at the base of most of the young papillomas, either in this experiment or in others in which non-destructive solvents and concentrations of carcinogenic agents were used. Although destructive inflammatory lesions in the dermis sometimes occur, they are not invariable or essential to the growth of papillomas nor are they specific for all carcinogenic chemicals.

### *Experimental observations*

The agents combined with simple mechanical injury in the present experiments were the following: (a) a carcinogenic chemical of medium potency, (b) a carcinogenic compound of low potency, (c) a single dose of a highly active carcinogenic compound, and (d) a very dilute solution of a highly active carcinogenic compound.

The potency or activity of the chemicals was judged by the proportion of animals which developed tumours when subjected to their action alone and not by the brevity of the latent period. The rate of induction of tumours is influenced by many factors besides the nature and dose of the carcinogenic chemical, some known, for example the solvents (Crabtree, 1940a), some probably unknown, the existence of which may be suspected from the observations of Crabtree (1940b, 1941). Incidence was judged to be increased by a single excision of skin if a tumour occurred at the site of a wound and nowhere else on that mouse. The methods used were similar to those previously described unless otherwise stated (Pullinger).

**Expt. A.I.** 1:2:5:6-*Dibenzanthracene*. Twenty-five young male Simpson mice were used. An area A, approximately 0.75 cm. in

diameter, was tattooed and the skin within it excised. After an interval of 2-4 weeks, which varied according to the rate and completeness of healing, painting was begun with a 0.1 per cent. solution of 1 : 2 : 5 : 6-dibenzanthracene in acetone. Three consecutive applications were made twice weekly because this compound is not soluble in acetone up to 0.3 per cent. Olive oil was applied when the skin became scaly. During the fifth month of treatment another area B, of the same size, was tattooed and excised. A pause was made in the painting to allow B to heal. Painting was then continued to the ninth month. Each mouse was numbered and records were made of all the tumours which appeared on A or B or elsewhere. Microscopic sections were made of all tumours, which were excised soon after they were seen.

*Result.* Twenty-one mice survived both excisions and 20 lived for nine months after the beginning of treatment. Fifteen mice developed tumours outside the tattooed sites. A papilloma appeared on the scar of the first excision (A) of one mouse only and nowhere else on the treated skin. Papillomas and sebaceous adenomas arose on areas A and B of five other mice but all five bore tumours elsewhere. The number of tumour-bearing mice was thus increased by one only, from 15 to 16 out of 21 (table).

TABLE  
*Effect of added trauma on tumour incidence*

Chemical compound applied	No. of mice used	No. alive for average latent period	No. of mice bearing tumours		Increase in no. due to wounds
			outside scars	on scars only	
Optimum dose of a weak carcinogen : 6-methyl-1:2-benzanthracene. Expt. A.II	31	19	2	0	0
Optimum dose of a carcinogen of medium activity: 1:2:5:8-dibenz- anthracene. Expt. A.I	25	21	15	1	1
Single dose of a highly active carcino- gen: methylcholanthrene. Expt. B. III					
½ mg.	50	28 (for 12 mths.)	2	0	0
1 mg.	48	45 (for 9 mths.)	1	0	0
5 mg.	48	46 (for 10 mths.)	0	1	1
Small repeated doses of a highly active carcinogen: benzpyrene 0·05 per cent. in acetone. Expt. IV	40	39	38	0	0
Optimum dose of a potent, rapidly acting carcinogen: 5:9:10-tri- methyl-1:2-benzanthracene. Expt. II (Pullinger, 1943)	30	30	22	4	4

(1941). By weekly painting with a 0.05 per cent. solution of benzpyrene in acetone Berenblum reduced the incidence of tumours in three trials to 6.5 per cent., 3 per cent. and nil respectively, in a test period of 20-22 weeks. This low yield was raised to 80 per cent. by the combined action of croton resin. The observations did not extend much beyond the 22nd week as a rule, but otherwise the conditions of his and the present experiments appear to have been comparable up to the time when excisions were begun. It may be argued that from then on the excisions exerted a stimulating effect and thus increased the total tumour incidence. From the figure, however, it will be seen that six papillomas occurred on the scarred areas A, and of these two were the first to appear on the mice that bore them; 7 papillomas appeared on the scarified areas B, of which 3 were the first to appear. If the element of chance is excluded for the purpose of the argument, wound healing localised and may have stimulated the 5 tumours which appeared first, leaving 33 to be attributed to the action of benzpyrene alone, that is 82 per cent. From the records of another experiment in which the same treatment with 0.05 per cent. benzpyrene was given but in which only one excision was made in each mouse and the tumours were left to develop, a similar result was obtained. Sixty-one out of 62 mice developed papillomas and carcinomas, only 6 of which appeared before the 21st week. Ten of the 61 appeared first on the scarred or scarified sites, leaving at least 51 to be accounted for by the action of benzpyrene alone, that is 65 per cent. It was this high incidence which led to a repetition of the experiment, taking all the precautions mentioned in expt. IV against concentration of the solution by evaporation.

As previously reported (Pullinger, expt. II), a highly active carcinogenic substance, 5:9:10-trimethyl-1:2-benzanthracene, was combined with wounding. The incidence of tumours due to this substance was originally given as 65 per cent. (Badger *et al.*, 1940). In expt. II, quoted above, it was found to be 74 per cent., due possibly to early excision of papillomas which might subsequently have regressed in accordance with all experience of the use of this compound. In addition 4 mice out of 30 developed papillomas on scars and nowhere else on the treated skin. These were left to develop and become established. They increased the incidence from 22 to 26 out of 30 mice or by 13 per cent.—the largest rise yet encountered.

### Discussion

Tumours are readily produced in rabbits by the combined action of two agents, one carcinogenic but used in a dosage which by itself would be ineffective, the other non-carcinogenic (Lacassagne; Lacassagne and Vincent; Burrows *et al.*; Burrows and Clarkson). The combined action of repeated burns of the skin of mice with a carcinogenic agent of low potency was also very effective (Rondoni



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## SHORT ARTICLES

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### A MEASURE OF THE STIMULATING EFFECT OF SIMPLE INJURY COMBINED WITH CARCINOGENIC CHEMICALS ON TUMOUR FORMATION IN MICE

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A suggestion has been made that during the course of skin painting with tar or other carcinogenic compounds many more cells become cancer cells than ever develop into tumours unless they receive a further stimulus to make them multiply. This further stimulus may be entirely non-specific in the sense that, acting alone, it never produces tumours (MacKenzie and Rous, 1941). Latent cancer cells capable of forming tumours were aroused to activity by simple wound healing in rabbit skin. The number of tumours occurring in the area of wound healing was sometimes three times the number that appeared in the same unit area on the rest of the skin. The present paper is concerned with an attempt to estimate the apparent stimulating effect of wound healing on mouse skin. Tumours have been localised in healing wounds and scars in mice painted with tar (Deelman, 1923-24) or synthetic chemical carcinogens (Pullinger, 1943) and some of these localised growths have appeared earlier than any others on the same mice. No estimate has been made of the part played by chance in this distribution. The best evidence that stimulation of latent neoplastic cells is a real factor in localisation is to be found in those mice which develop tumours on the scars alone and nowhere else on the painted skin (Pullinger, 1945). It is reasonable to suppose that in these mice chance has played no part, and some factor in wound healing has provided the added impulse. In rousing these latent neoplastic cells of refractory mice to multiply, the healing of single wounds is probably less effective than more prolonged influences. In estimating its effect in highly susceptible mice which in any case grow tumours without additional stimulation, the element of chance comes into play and requires to be measured.

In the first place an estimate was made of the percentage of tumours localised, whether by chance or by the effects of wounding. This was based on the results of several experiments in which optimum doses of very active carcinogenic chemicals had been used. These results are grouped in table I and compared with similar experiments with optimum doses of less active compounds or with very small doses of benzpyrene or methylcholanthrene. The dose of benzpyrene in the latter group was sufficient to give a tumour incidence of 97 per cent., but the latent period was so extended that any influence due to wound healing may have been lost. The placing of this experiment among those with the less active compounds and less effective dosages is perhaps arbitrary, but the result is consistent with the others. In all four experiments with large doses of active compounds the number of tumours localised on scars was always about half of all the tumour-bearing animals and the average exactly 50 per cent. Among the less active compounds the percentage varied from nil to six.



To estimate the proportion due to chance another experiment was done. The matter could not be treated abstractly, because the sites where tumours appear are irregular, sometimes extremely so, and subject to unknown factors (Law, 1941); these sites are frequently outside the areas deliberately treated and even the treated areas cannot be accurately defined because the solutions flow to different limits at each painting; mouse skin is extremely elastic and

TABLE I  
*Percentage incidence of tumours on excised sites*

Optimum doses of highly active carcinogenic chemicals			
Carcinogenic compound and dose used	No. of mice	No. of tumours on excised sites	Total no. of mice with tumours
0.3 per cent. benzpyrene in liquid paraffin. (Expt. I, Pullinger, 1943)	10	8	10
0.05 per cent. 5:9:10-trimethyl-1:2-benz-anthracene. (Expt. II, Pullinger, 1943)	30	14	26
0.05 per cent. benzpyrene in acetone twice weekly, unmeasured dose, S. XVIII	22	11	22
0.05 per cent. benzpyrene in acetone once weekly, S. XVII, unmeasured dose	30	13	30
Totals . . .	92	46	88
Percentages . . .	...	50	95
Less active compounds or small doses of active ones			
1:2:5:6-dibenzanthracene = 0.3 per cent. in acetone. (Expt. A.I, Pullinger, 1945)	21	6	15
6-methyl-1:2-benzanthracene = 0.3 per cent. in acetone. (Expt. A.II, Pullinger, 1945)	19	0	2
Methylcholanthrene, single doses. (Expt. B.III, Pullinger, 1945)	119	1	4
0.05 per cent. benzpyrene; 0.1 c.c. in acetone once weekly. (Expt. IV, Pullinger, 1945)	39	6	38
Totals . . .	198	13	59
Percentages . . .	...	3	20.8

the animals increase in size during the course of an experiment. Conditions were made comparable with those in previous experiments with the exception that a ring (A) of the same size as was made in previous experiments was tattooed several days before painting was begun. No excision was made. The area was only marked. Any possible influence due to the presence of india ink in the subcutaneous tissues was the same in all experiments. The scarring which resulted from the temporary presence of linen threads in the subcutaneous tissue of mice was found by Orr (1934, 1935) to accelerate tumour induction but not to increase the incidence of tumours.

Numbers were tattooed on the ears and diagrammatic records kept of all animals which developed tumours on the tattooed sites. As in previous experiments each tumour was excised soon after it appeared. Of 107 mice which survived tattooing and painting, 91 developed tumours (85 per cent.) and 29 bore tumours on the marked areas. Thus 31 per cent. were localised by chance, that is, more than half the number (50 per cent.) localised in experiments combined with excisions. Of these 29 tumours localised by chance at an arbitrary site, 13 appeared there before any others on the same mice, or 45

per cent as compared with 65 per cent when excisions were made. The difference in incidence on the marked and excised sites is insufficient to attribute it to any factor other than chance and the same conclusion applies to earlier incidence.

A review of the experience of other authors who combined small doses of carcinogenic agents with non specific agents led to the conclusion that the action of the second or non specific agent required to be repeated or prolonged after the manner of a chronic irritation or inflammation (Pullinger, 1945). A similar conclusion was reached by Berenblum (1944). The amount of the carcinogenic agent may then be so small as to be ineffective alone. It might similarly be found that repeated wounding would have a greater stimulating effect than a single excision. In many of the experiments in which tumours grew on tattooed sites from which excisions had been made, these tumours were subsequently removed. They were always examined microscopically to judge both of their nature and complete removal. Despite complete removal a second, third or fourth tumour sometimes arose on the same site (Pullinger, 1943). When this happened it gave a strong impression of local stimulation, but there is one objection to such an assumption. As mentioned by MacKenzie and Rous, skin heals by epithelial migration from the injured edge. Multiplication occurs at some distance away, where, according to Wigglesworth (1937), cell scarcity may provide the effective impulse to divide. That epithelial migration occurs even in tar treated tumour bearing skin was demonstrated in macroscopically visible form by Rous and Kidd (1941). If this is the case then an area which is repeatedly excised becomes a focus to which potentially malignant cells from surrounding skin are continually migrating. At this focus they collect among the hyperplastic but non malignant cells. The fate of hyperplastic squamous epithelial cells, like normal ones, is to mature, die and be cast off, while that of malignant ones is to multiply and form tumours, thereby revealing themselves and maintaining their existence in the body. These malignant cells now concentrated at a focus might equally well have multiplied in these mice had they remained undisturbed at their original pre migration sites. The tumours which grow from them at the hub of the migration area cannot be attributed to stimulation alone. Concentration may be another factor and both may play a part.

Evidence of stimulation from repeated injury was sought in another way. Records of several experiments were compared to determine the average number of tumours which made their appearance at four weekly intervals up to the seventh month after the end of painting. In some of these experiments multiple excisions had been made to remove young papillomas, in others only one excision of a tattooed site A (Pullinger, 1943, 1945). The seventh month was chosen as the limit of time for comparison because few mice in which tumours are allowed to develop survive longer than this. A much larger number survive to the seventh month or longer when young papillomas are regularly excised. The results are compared in table II. When one excision only was made (area A in series XVII) there was no increase in the average number of tumours borne by each mouse from the third to the seventh month. This may have been due in part to an exceptionally high rate in the third month, but whereas in all other experiments there was a gradual rise, in this case there was none. When multiple excisions had been made, whether the dose of the carcinogenic chemical was high or low, there was in the seventh month an increase of two to three times the average number of tumours over the third month, with a continuous rise in the intervening months. This increase is comparable with that obtained by MacKenzie and Rous. It is the more remarkable in that with each excision the total number of cells originally exposed to the carcinogenic chemical is diminished and there is a replacement by normal cells from the periphery. A dilution of potentially

neoplastic cells is going on, but at the same time the number of tumours is increasing. The rate of increase becomes so rapid that excisions are no longer practicable and the mice have to be killed (see, for example, fig. 1, Pullinger, 1945).

TABLE II

*Increase in tumour incidence per mouse due to multiple excisions*

Carcinogenic compound and dose used	No. of months after end of treatment					Increase at 7th month
	3	4	5	6	7	
0.05 per cent. benzpyrene once weekly, unmeasured, S. XVII. Single excision						
No. of mice with tumours . . . . .	10	5	5	10	22	
Total no. of tumours . . . . .	23	10	11	23	50	
Average no. of tumours per mouse . . . .	2.3	2	2.2	2.3	2.1	Nil
0.05 per cent. benzpyrene once weekly, 0.1 c.c. (Expt. IV, Pullinger, 1945.) Multiple excisions						
No. of mice with tumours . . . . .	22	24	28	31	36	
Total no. of tumours . . . . .	33	48	71	85	113	
Average no. of tumours per mouse . . . .	1.5	2	2.6	2.7	3.1	2 times
0.05 per cent. benzpyrene twice weekly, unmeasured, S. XVIII. Multiple excisions						
No. of mice with tumours . . . . .	6	15	17	19	20	
Total no. of tumours . . . . .	10	27	47	72	96	
Average no. of tumours per mouse . . . .	1.6	1.8	2.1	3.7	4.8	3 times
0.3 per cent. benzpyrene in paraffin or olive oil, S. XX. Multiple excisions						
No. of mice with tumours . . . . .	6	8	10	11	11	
Total no. of tumours . . . . .	13	22	35	48	65	
Average no. of tumours per mouse . . . .	2.1	2.7	3.5	4.3	5.9	2.8 times

It is difficult in mice to devise an entirely satisfactory method for estimating a possible stimulating action. The one just described is merely an approximation. Among a number of mice painted with carcinogenic chemicals it is often the case that early tumour-bearers die or have soon to be killed. These may also be the most susceptible mice which, if they lived, would produce the greatest number of tumours per mouse. In a similar group from which excisions are made, the most susceptible mice are enabled to live on, even for their natural span of life. Experimental conditions are therefore never quite comparable.

### Conclusions

Multiple simple excisions from mouse skin treated with small or large doses of benzpyrene stimulated the formation of tumours to approximately two to three times the number obtained in susceptible mice from which only one excision was made.

The results tend to confirm the general proposition of MacKenzie and Rous that tar or other carcinogenic agent renders many more epidermal cells neoplastic than ever declare themselves by forming tumours. In mice, however, the potentiality is not so readily disclosed as in the rabbit. Multiple injuries are more effective in revealing it. These multiple injuries provide many physical factors. Some factors are purely epidermal in origin, for example epithelial migration and multiplication; others are inflammatory—in the connective tissue—or ischaemic, as suggested by Orr. All combined are likely to provide

a state of chronic irritation or reaction to repeated injury. No clue was found to indicate precisely which of the many factors provided the effective non-specific stimulus.

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# COR BIATRIUM TRILOCULARE WITH TRANSPOSITION OF THE ARTERIAL TRUNKS A RARE CONGENITAL CARDIAC MALFORMATION

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A short description is given below of an unusual congenital cardiac malformation. The subject was a female child aged one year who had shown a moderate degree of cyanosis from birth. About two months prior to death, the child had become more cyanotic and liable to fits of screaming, the fits became more frequent and lasted from 1 to 3 hours. At first relief followed the inhalation of amyl nitrite but later no drug appeared to alleviate the condition to any appreciable degree. The pulse was full and regular in force and rhythm. Systolic and mid diastolic murmurs were heard over the mitral area and a systolic murmur at the aortic area was conducted down the sternum.

At autopsy the cause of death was found to be thrombosis of the sagittal and lateral sinuses and beyond the cardiac malformation no other abnormalities were found.

The heart weighed 59 g, it showed transposition of the arterial trunks and the division of its cavity into five chambers—two atria and two ventricles plus an accessory ventricle. The right atrium received the systemic blood by way of the superior and inferior vena cavae. The left atrium received the pulmonary blood by way of the pulmonary veins. The foramen ovale was patent but small (diameter 5 mm).

The ventricular system consisted of three chambers, two large and one small, the latter, the accessory ventricle, being placed antero superior to and between the other two. The arrangement showed a resemblance to the ventricular system in the heart of the python (*P. molurus*) as described by Abbott (1936). In the heart under discussion the right ventricle (fig. 1) corresponded to the cavum venosum of the python heart, the left ventricle (fig. 2) to the cavum arteriosum and the accessory ventricle (fig. 3) to the ventral ventricle. The right and left ventricles communicated with each other by an interventricular foramen. The accessory ventricle communicated with the right ventricle only, instead of with both right and left ventricles, as in the heart of the python, in

which the ventral ventricle communicates with the cavum venosum through a gap in the bulbar septum, and with the cavum arteriosum through a hole in the floor of the ventral ventricle. A further similarity, however, existed in that the pulmonary artery arose from the accessory ventricle and its valve was, like that of the python, bicuspid. The parallel has been drawn to simplify the description of the main abnormalities presented.

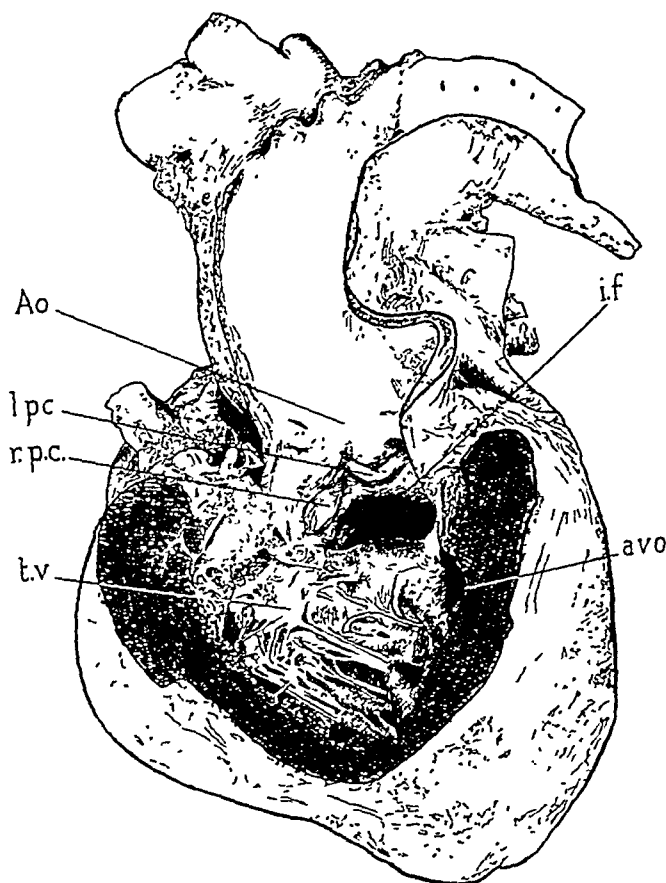


FIG. 1.—Right ventricle laid open showing the aorta (Ao.) and its left posterior cusp (l.p.c.), with the orifice of the right coronary artery and the right posterior cusp (r.p.c.) (the anterior cusp is obscured), the interventricular foramen (i.f.), the tricuspid valve (t.v.) and the opening between the right ventricle and the accessory ventricle (a.v.o.).

Furthermore, with the transposition of the arterial trunks, the aorta arose from the right ventricle. The cusps of the aortic valve were arranged one anterior, one left and one right posterior. The left coronary artery took its origin from the sinus of the anterior cusp, the right coronary from the sinus of the left posterior cusp. These findings would indicate arrest or delay of the clockwise torsion which, according to the theory of Spitzer (1923), takes place in the growth of the primitive embryonic heart between its fixed arterial and venous ends during the process of septation. In this instance, therefore, the anterior cusp of the aortic valve corresponds to the normal right cusp and the left posterior cusp to the normal left cusp.

The ductus arteriosus was closed at its pulmonary end by a thin membrane.

The septum between the right and left ventricles was well formed except for the deficiency at its upper end, giving an interventricular foramen 9 mm. broad by 8 mm. long. The left ventricular aspect of the septum sloped towards this defect, giving the impression that during ventricular systole the blood in the left ventricle would be directed upwards and to the right through the interventricular foramen into the aorta. This was further borne out by the

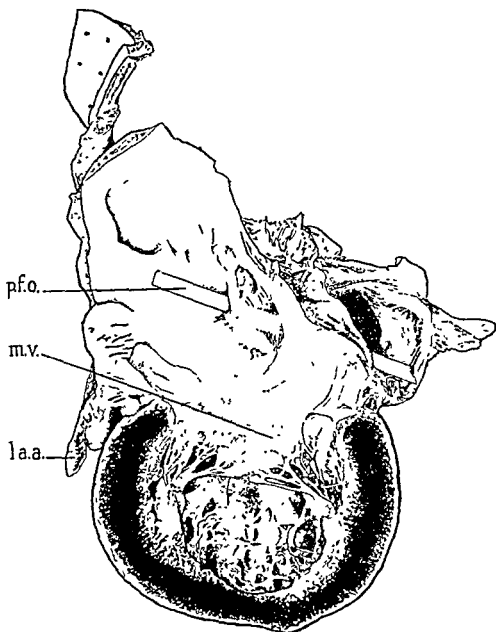


FIG. 2.—Left atrium and ventricle displayed showing the mitral valve (m.v.), a probe through the patent foramen ovale (p.f.o.), and the left auricular appendage (l.a.a.).

fact that the mitral valve was so placed that when its curtains were in apposition, as in ventricular systole, it would act like a paddle directing the blood column towards the interventricular foramen.

In the right ventricle the atrio-ventricular orifice was situated to the right of the aortic orifice and separated from it by the tricuspid valve. The upper edge of the defective interventricular septum possessed a rolled-over margin which projected into the cavity of the right ventricle, making the communication between the right ventricle and the aorta narrower than the illustration (fig. 1) indicates.

The right ventricle and the accessory ventricle communicated at the apex of a cone-shaped vestibule and the opening, 5 mm. in diameter, so formed was

divided from above downwards by a column of membranous tissue. During ventricular systole the contents of the right ventricle would appear to be directed towards the accessory ventricle via the vestibule and so, in the main, into the pulmonary artery. As this opening is much narrower than the normal exit from the right ventricle via the pulmonary artery, it is not surprising that the wall of the right ventricle measured 8 mm. in thickness, that of the left ventricle

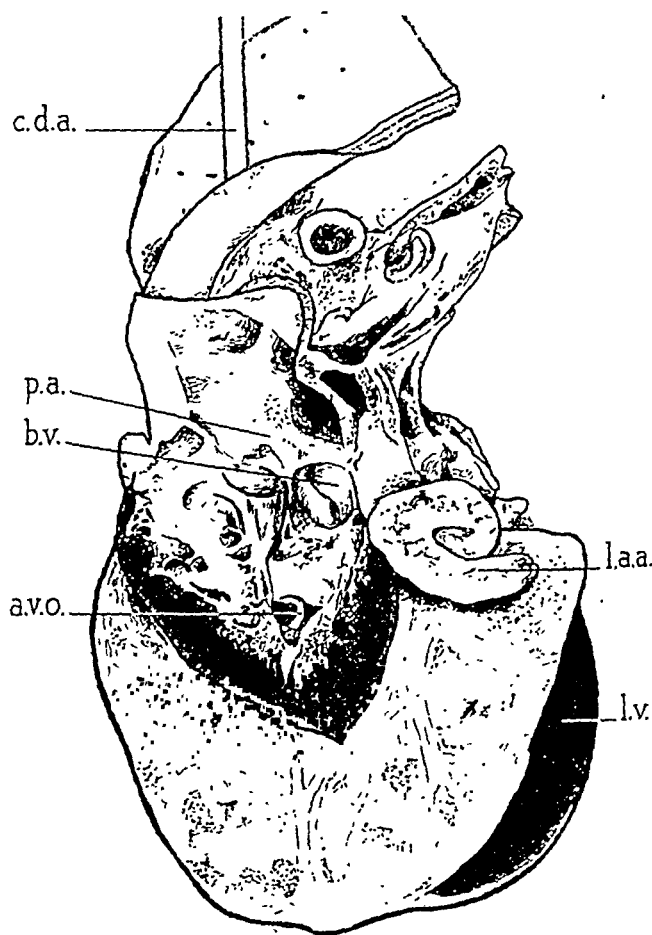


FIG. 3.—Accessory ventricle exposed to show the pulmonary artery (p.a.) with its bicuspid valve (b.v.), the opening between the right ventricle and the accessory ventricle (a.v.o.) with its membranous band, the left auricular appendage (l.a.a.), a probe in the closed ductus arteriosus (c.d.a.) and the incision into the left ventricle (l.v.).

only 6 mm. That admixture of arterial and venous blood occurred through the patent foramen ovale is almost certain, and, while the possibility of some mixing of the bloods in the right and left ventricles resulting from the inter-ventricular septal defect cannot be denied, the circulation indicated above would appear to be the most likely as well as the most desirable if a relatively satisfactory circulation was to be maintained.

The details of the heart are shown in figs. 1-3. These figures are accurate in detail and in proportion, having been made according to the method of Shennan (1929).

Reference to available literature has failed to reveal any record of a similar cardiac anomaly. The cardiac malformation which most closely approximates to the present one is the cor batrium trilobulare originally described by Holmes and re-published by Abbott (1936). In Holmes's case, however, the inter-ventricular septum is absent and the arterial trunks are not transposed.

A diagram, after Spitzer (1923), showing the division of the ventricles and the relative position of the arterial ostia in the heart under discussion is given (fig. 4). From this it would appear that the communication between the right

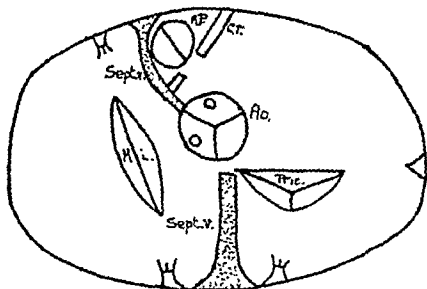


FIG. 4.—Diagram after Spitzer showing the interventricular septum (Sept. v.), crista supraventricularis (c.r.), mitral valve (Mi.), tricuspid valve (Tric.), pulmonary artery (A.P.) and aorta (Ao.).

ventricle and the accessory ventricle is through a defect in the crista supraventricularis.

The author wishes to thank Professor D. F. Cappell for facilities and advice and Dr J. Thomson for clinical data.

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576 . 8 . 097 . 3 : 576 . 858 . 13 (*Vaccinia*)

#### THE VIRUS-NEUTRALISING POWER OF SERUM FROM RECENTLY VACCINATED PERSONS

J. F. LOUTIT and D. McCLEAN

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Elstree, Herts*

With the recent developments in drying from the frozen state it is possible to store human serum for long periods without deterioration. It may be desirable to collect and dry serum from recently vaccinated persons for trial



divided from above downwards by a column of membranous tissue. During ventricular systole the contents of the right ventricle would appear to be directed towards the accessory ventricle via the vestibule and so, in the main, into the pulmonary artery. As this opening is much narrower than the normal exit from the right ventricle via the pulmonary artery, it is not surprising that the wall of the right ventricle measured 8 mm. in thickness, that of the left ventricle

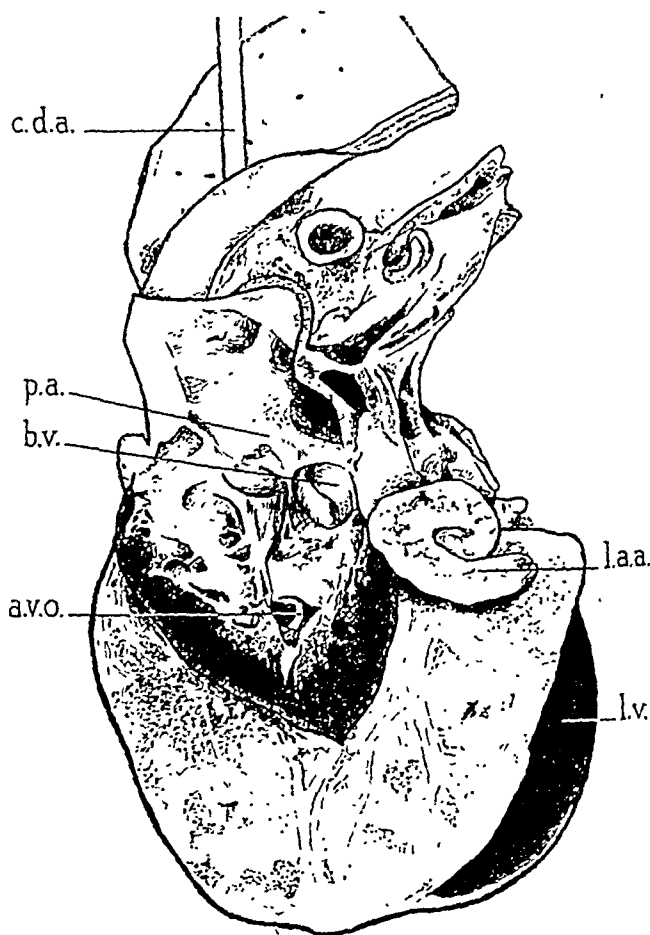


FIG. 3.—Accessory ventricle exposed to show the pulmonary artery (p.a.) with its bicuspid valve (b.v.), the opening between the right ventricle and the accessory ventricle (a.v.o.) with its membranous band, the left auricular appendage (l.a.a.), a probe in the closed ductus arteriosus (c.d.a.) and the incision into the left ventricle (l.v.).

only 6 mm. That admixture of arterial and venous blood occurred through the patent foramen ovale is almost certain, and, while the possibility of some mixing of the bloods in the right and left ventricles resulting from the inter-ventricular septal defect cannot be denied, the circulation indicated above would appear to be the most likely as well as the most desirable if a relatively satisfactory circulation was to be maintained.

The details of the heart are shown in figs. 1-3. These figures are accurate in detail and in proportion, having been made according to the method of Shennan (1929).

Reference to available literature has failed to reveal any record of a similar cardiac anomaly. The cardiac malformation which most closely approximates to the present one is the cor biatrium triloculare originally described by Holmes and re-published by Abbott (1936). In Holmes's case, however, the inter-ventricular septum is absent and the arterial trunks are not transposed.

A diagram, after Spitzer (1923), showing the division of the ventricles and the relative position of the arterial ostia in the heart under discussion is given (fig. 4). From this it would appear that the communication between the right

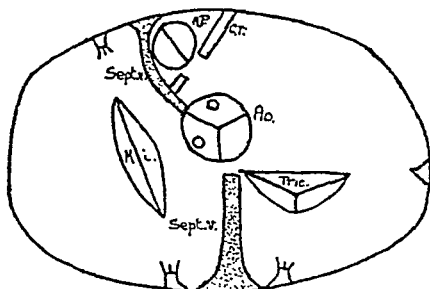


FIG. 4.—Diagram after Spitzer showing the inter-ventricular septum (Sept. v.), crista supraventricularis (c.r.), mitral valve (M.V.), tricuspid valve (Tric.), pulmonary artery (A.P.) and aorta (A.O.).

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The author wishes to thank Professor D. F. Cappell for facilities and advice and Dr J. Thomson for clinical data.

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576 . 8 . 097 . 3 : 576 . 858 . 13 (Vaccinia)

#### THE VIRUS-NEUTRALISING POWER OF SERUM FROM RECENTLY VACCINATED PERSONS

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With the recent developments in drying from the frozen state it is possible to store human serum for long periods without deterioration. It may be desirable to collect and dry serum from recently vaccinated persons for trial

in the treatment of complications following vaccination and, possibly, of smallpox itself. In the absence of precise information, preliminary observations have been carried out to decide the time after vaccination at which the highest titre of virus-neutralising antibody is attained.

### *Methods*

Blood was collected from donors at different periods after ordinary Jennerian vaccination and the resulting serum pooled in groups as shown in tables I and II. In the first test, a sample of dried serum from a single individual bled after 28 days (kindly supplied by Brigadier Sir Lionel Whitby) and hyperimmune rabbit serum obtained after immunisation with elementary body suspensions from a strain of vaccinia repeatedly passed on the rabbit skin were tested in parallel with the serum pools. Twofold dilutions of the human sera and fivefold dilutions of the hyperimmune rabbit serum were mixed with an equal volume of a fixed dose of a vaccine lymph which has been used for many months as a laboratory standard for comparison with other lymphs. Similar mixtures of the lymph with dilutions of normal rabbit serum were made to control the activity of the lymph on each test animal. After one hour at room temperature and one hour in the refrigerator, the mixtures were tested by scarification on the clipped backs of three rabbits and the resulting vaccinal lesions observed on the 5th day. In the second test, in which one rabbit only was used, the titration with normal rabbit serum was omitted and, instead, the vaccine lymph was inoculated alone at the test dose level and at tenfold and one hundredfold dilutions of this in order to gain some idea of the number of infective doses of virus neutralised by the serum samples. Inoculation of dilutions of standard lymph, either alone or mixed with normal serum, also served to control any variation in the susceptibility of individual rabbits to the virus. Experience has shown that, provided a normal reaction is obtained with constant dilutions of a laboratory standard lymph, titrations of unknown virus preparations on different animals can be directly compared.

### *Results*

The results of the first test are shown in table I. It is perhaps surprising that serum pool A showed slight virus neutralising power; this may have been due to the residual antibody from previous vaccination of one or more donors. Serum pool B, however, showed little or no neutralising activity. When allowance is made for variations in results that are likely in a test as crude as the one employed, it appears that serum pools C, D and E are all of about the same potency. Since the titre of these sera was so much higher than those of pools A, B and F and they were all collected between the 12th and 22nd day, it is not of much practical importance that their end-point was not reached in this test. Although serum FLB, from an individual donor bled after 28 days, showed similar potency to pools C, D and E, sample F from eight donors bled at approximately the same interval, showed a definite fall in neutralising activity. It will be noticed that the hyperimmune rabbit serum caused substantial neutralisation of virus up to 1:6250. These results suggested that serum with the highest titre is likely to be obtained during the third week after vaccination but a fall in potency may occur by the end of the fourth week.

In order to confirm this result and to determine whether any fall in neutralising potency occurred at a longer period after vaccination, a further test was made on pools of serum collected at the end of the third and fourth weeks and during the sixth week. The results are shown in table II. All three samples of serum gave lower neutralising activity than those collected during the third and fourth weeks for the previous test. Virus neutralisation by all

TABLE I

*Neutralisation of vaccinia virus by pooled human sera  
after vaccination*

No of rabbit	1		2			3			
Serum pool	A	B	C	D	E	F	FLB	Hyperimmune rabbit serum	
No of donors	9	3	3	9	4	8	1		
Days after vaccination	3 5	8 10	12 13	17	19 22	24 28	28		
Dilution of serum								Dilution of serum	
1 2	3	sc	0	2	1	1	0	1 2	0
1 4	8	sc	1	7	1	1	1	1 10	1
1 8	4	sc	1	4	0	c	3	1 50	0
1 16	sc	sc	6	6	1	sc	3	1 250	0
1 32	c	c	1	2	1	sc	3	1 1250	1
1 64	c	c	0	3	6	sc	7	1 6250	3
Dilution of normal rabbit serum									
1 2	c			c			sc		
1 4	sc			c			c		
1 8	c			sc			sc		

Test dose of vaccine lymph = dilution of  $10^{-3}$

c = confluent vaccinal eruption

sc = semi confluent vaccinal eruption

numerals = number of discrete vesicles

TABLE II

*Neutralisation of vaccinia by pooled human sera after vaccination*

Serum pool	G	H	I	Titration of test lymph	
No of donors	7	7	5	Dilution of lymph	
Days after vaccination	20 23	25 29	35 43		
Dilution of serum					
1 2	0	4	0	$10^{-3}$	c
1 4	4	4	0	$10^{-4}$	sc
1 8	2	7	3	$10^{-5}$	5
1 16	12	20	6		
1 32	sc	sc	c		
1 64	sc	c	c		

Test dose of vaccine lymph = dilution of  $10^{-3}$

c = confluent vaccinal eruption

sc = semi confluent vaccinal eruption

numerals = number of discrete vesicles

three sera is, however, quite definite at a dilution of 1:16 and there is no indication of a fall in activity up to 35-43 days after vaccination. This test in rabbits was repeated with the same result.

The only explanation that can be offered for the weak neutralisation by serum F in the first experiment is that this pool may have contained the serum of more than one poor reactor to vaccination; it is unlikely that serum pools C-I all contained an abnormal proportion of good reactors. The relative failure of pool F indicates that it is essential to test each pool for virus-neutralising activity and, from this point of view, pools from a large number of donors would be most practical. On the other hand, jaundice is known to follow the administration of serum and it is logical, in the present state of knowledge, to collect pools from a small number of donors (say ten) to lessen the risk of contaminating a large pool with one icterogenic serum.

Although 5-20 ml. of convalescent serum have been given in the past, it is likely that larger doses may be given in the future. Therefore, in order to avoid the rare but undesirable reactions from high hæmagglutinin titres, it would be desirable for each pool to contain serum from group A and group B or from group AB blood.

### Conclusion

Tests on the virus-neutralising activity of serum from recently vaccinated persons indicate that the most potent sera are likely to be obtained during the third and fourth weeks after vaccination but that no considerable fall in potency occurs up to the end of the sixth week.

Despite the risk of fluctuation in neutralising activity, it is suggested that pools from a relatively small number of donors (*e.g.* ten) should be kept separately and that each pool should be tested for neutralising potency; thus the risk of contaminating a large volume with one icterogenic serum will be avoided. Precautions for the avoidance of possible reactions due to a high hæmagglutinin titre of the serum are described.

616 . 24—092 . 9 (*Rattus*): 549 . 514 . 5<sup>2</sup>

## THE EFFECT OF OLIVINE ON THE LUNGS OF RATS

E. J. KING, NANCY ROGERS and MARGARET GILCHRIST

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and

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(PLATE LXVI)

Attempts to prevent silicosis in modern industry have, in general, taken three lines: (1) dust suppression by controlled ventilation and the introduction of water at the site of dust formation; (2) the use of filter or positive pressure masks; and (3) the substitution of non-siliceous materials for quartz in some processes. Two outstanding examples of the latter are the modern use of powdered alumina in place of quartz for bedding the china bodies in the pottery industries and the substitution of steel shot for quartz in the sand-blasting

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of castings. This process of cleaning and polishing an iron or steel object, cast from the molten metal, by means of a coarse abrasive powder blown at it under high pressure is known as "blasting". It remains one of the most dangerous processes in the foundry industry in spite of the use of steel shot instead of sand, because of the adherence of particles of quartz to the metal body from the sand mould in which it is cast. Another dangerous process consists in removing adhering nests of sand by means of pneumatic or hand tools, a process known as "fettling". The substitution of some non-silicosis-producing material in the moulding of metal castings might well result in a lessening of the silicosis risk in the foundries.

Certain varieties of the mineral olivine are highly refractory substances, consisting essentially of forsterite (magnesium orthosilicate— $\text{Mg}_2\text{SiO}_4$ ), subordinate amounts of fayalite (ferrous orthosilicate— $\text{Fe}_2\text{SiO}_4$ ) and very minute amounts of the orthosilicates of manganese, nickel and cobalt. The refractory properties of olivine rock were discovered in 1925 by Goldschmidt, who suggested and introduced the use of olivine rock (dunite) as an industrial refractory (Goldschmidt, 1938, 1940; see also Harvey and Birch, 1938). Goldschmidt and Stenvik (1928, unpublished industrial experiments in Norway on the use of olivine as a refractory: British patent 321609, 1929) had already made industrial experiments on the use of olivine sand and of crushed dunite rock instead of quartz sand for making moulds for foundries. Elstad and Stenvik (unpublished observations) have carried out animal experiments in Norway since 1940 to ascertain whether or not the inhalation of olivine dust might involve a silicosis hazard, as industrial olivine contains about 42 per cent. by weight of combined silica and is easily decomposed even by very weak acids. Reports of these tests have not yet been published so far as is known.

Technical experiments in Norway on the use of crushed olivine rock in foundries have been carried out on a large scale since 1935 and have given very satisfactory results as regards quality of castings. In olivine moulds, not only steel but also stainless ferro-alloys having high temperatures of fusion give excellent castings with remarkably clean surfaces. Not only is the silica dust hazard of handling the ordinary mould materials avoided, but also the equally dangerous operation of cleaning the surface of castings by means of fettling and sand-blasting with quartz either eliminated or greatly reduced. Full reports of these technical experiments will be published elsewhere (Goldschmidt).

### Methods

The percentage composition of the sample of olivine used was as follows:— $\text{SiO}_2$  41.42,  $\text{MgO}$  51,  $\text{FeO}$  5.5,  $\text{Fe}_2\text{O}_3$  0.3,  $\text{Cr}_2\text{O}_3$  0.4,  $\text{Al}_2\text{O}_3$  0.2,  $\text{MnO}$  0.1,  $\text{NiO}$  0.4,  $\text{CoO}$  0.02,  $\text{CaO}$  0.00,  $\text{K}_2\text{O} + \text{Na}_2\text{O}$  0.01,  $\text{Ti}_2\text{O}$  and  $\text{P}_2\text{O}_5$  0.00; ignition loss 0.4-0.5. A mineralogical determination of the composition of the sample reveals in addition to the main constituent—the orthosilicate olivine (92 per cent. by weight)—about 5 per cent. of enstatite and magnesian amphibole and small traces of the minerals phlogopite, chlorite and corundum, about 1 per cent. of oxydic iron ores (chromite and magnetite) and about 2 per cent. of hydrated secondary magnesian silicates (essentially serpentine). When treated alternately with acid and alkaline solutions, the olivine is completely dissolved, leaving a residue mainly consisting of enstatite (essentially  $\text{MgSiO}_3$ ) and chromite (essentially  $(\text{Fe}, \text{Mg}) (\text{Cr}, \text{Fe}, \text{Al})_2\text{O}_4$ ). A sample of such olivine rock of particle size  $< 2 \mu$  was prepared by crushing, grinding and sedimentation in water.

For the purpose of injecting into animals, 750 mg. of powdered olivine were weighed into a 50 ml. conical flask and suspended in 30 ml. of isotonic sodium chloride solution. The flask was closed with a rubber cap through which a fine hypodermic needle was inserted (to allow escape of air). The flask, saline, rubber cap and needle had previously been sterilised by boiling. The mixture was heated on a boiling water-bath for 15 min. and was then used, immediately

after cooling, for introduction into the lungs of black and white rats, by Kettle and Hilton's method of intratracheal insufflation. The rats were anaesthetised with ether in a bell jar and the hair was shaved from the neck. A longitudinal incision of about 1 cm. was made and the trachea exposed by blunt dissection. A short needle of 12 gauge fitted to a 5 ml. syringe was inserted through the wall of the trachea and 2 ml. of the inoculum (50 mg. olivine) were injected. The suspension of olivine was shaken in the flask as the 2 ml. were being withdrawn and again gently in the syringe until just prior to insertion of the needle. The needle was immediately withdrawn and the wound closed with a single cat-gut suture. The procedure is described in detail by Belt and King (1945). Of nine rats so treated none failed to survive the operation; they lived for the periods shown below. At the end of five months the four survivors were killed with coal gas and the experiment terminated.

The lungs of all the animals were removed and fixed by injecting formal-saline mixture into the trachea, which was then tied and the organs suspended in the fixative. Serial paraffin sections were stained with haematoxylin and eosin, van Gieson, and Gordon and Sweets's reticulin stain. Immediately adjacent sections were subjected to micro-incineration.

#### *Survival of rats after administration of olivine*

Of the 9 rats used in the experiment, one was killed at the end of a fortnight, 4 died during the next four months and 4 were alive at the end of five months. The numbers are too small to say if this is a normal rate of mortality or not.

#### *Histology*

As a result of the various time intervals at which the experiments were terminated it was possible to study the course of the lesions produced in the lungs. The earliest reaction to the dust was predominantly koniophage in type, consisting of an outpouring of large phagocytic cells into the alveoli. These cells phagocyted the olivine particles and a number of air sacs were filled with them. A small number of the olivine-containing macrophages were also present in the lymphatics of the alveolar wall.

In the early lesions there was also a slight polymorphonuclear leucocytic reaction. Most of these cells showed degenerative changes, with pyknotic nuclei and breaking down of their cytoplasm. There were also areas of congestion, slight emphysema and collapse (fig. 1).

Gradually as the duration of the experiments increased the proportion of alveoli containing koniophages diminished and the proportion of these cells in the alveolar wall lymphatics and to a slight degree in the peribronchial and perivascular lymphoid tissue increased. The alveolar walls of the older lesions showed well marked thickening due to dust-containing macrophages in the lymphatics, congestion and slight fibrosis (fig. 2). The koniophages showed a gradually increasing content of brownish pigment granules which gave a strongly positive Perl's reaction for iron. Olivine crystals themselves give a negative reaction, but in the experiment of 14 days' duration, a number of the macrophages contained iron-reacting pigment granules, and some of the phagocyted olivine crystals took up the blue stain. These findings suggest that the olivine, after contact with tissue fluids, gradually undergoes partial solution, with liberation of iron-containing pigment granules; the crystalline particles also diminish in size.

The changes produced in the lymph nodes are slight and consist of an increase in reticulin fibres with cellular increase and fibrosis.

The reaction to olivine is thus of simple foreign body type. There is no organisation with reticulin fibrosis. The dust appears to be non-toxic in comparison with silica.

## OLIVINE: A QUARTZ SAND SUBSTITUTE

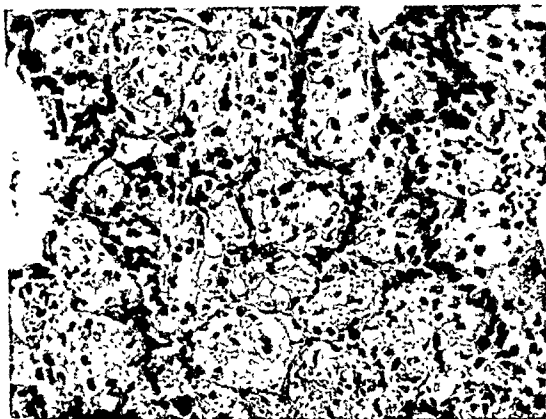


FIG. 1.—Section of rat lung 14 days after intratracheal insufflation with olivine. The air sacs contain numerous pale-staining macrophages which have phagocytosed the olivine particles. Polymorphonuclear leucocytes are also present in the exudate. These cells show moderate degenerative changes, with pyknotic nuclei.  $\times 315$ .

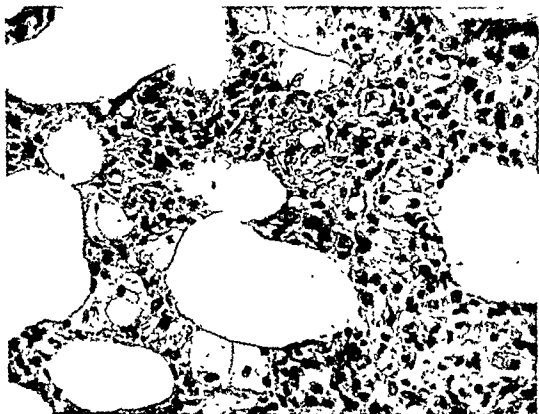


FIG. 2.—Section of rat lung 160 days after intratracheal insufflation with olivine. The majority of the air sacs are empty, but the alveolar walls are thickened, owing to the presence of dust-containing macrophages in the lymphatics, congestion and slight fibrosis.  $\times 315$ .





## Summary

Magnesian olivine is a highly refractory silicate mineral. Rock consisting essentially of such olivine (dunite) occurs particularly in Norway and has been used successfully in the foundry industry as a substitute for quartz sand. Its effect on the lungs of rats has been tested by intratracheal insufflation. Only a minimal reaction of simple foreign-body type appears to be produced.

Our thanks are due to Professor J. H. Dible for his helpful criticism and advice, to Mr W. Weedon and Mr A. Hoffer for their assistance with the experiments and their care of the animals and to Mr J. Baker and Mr J. Griffin for their preparation of the histological material.

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616 . 24—003 . 656 . 6—021 . 6 (Rattus)

# A COMPARISON OF THE EFFECTS OF LÆVO-ROTATORY AND DEXTRO-ROTATORY QUARTZ ON THE LUNGS OF RATS

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(PLATE LXVII)

Many natural substances occur in either the dextro- or lævo-rotatory form. The amino acids of animal tissues, for instance, are present as lævo-rotatory isomers. Certain compounds of pharmacological interest are more active in one optical form than the other. Quartz also may occur in two antipodic forms, as right and left quartz. Here the activity is due to the arrangement of the atoms in the solid crystal only and is lost when the silica is in solution. It seemed possible that the pathogenic action of quartz might differ with the dextro- or lævo- arrangement as evidenced by optical activity.

## Methods

Plates cut parallel to the base of quartz crystals were selected by means of microscopic tests for right or left rotation and freedom from dextro-lævo twinning, and one of each was used. Small chips mounted in canada balsam



*Summary*

Dextro- and lævo-rotatory quartz administered by Kettle and Hilton's insufflation method bring about similar changes in the lungs of rats. Their different antipodic structural configuration appears to have no effect on the experimental lesion produced.

We would like to express our thanks to Professor Goldschmidt for suggesting this investigation and for helpful comments. Our thanks are due to the Medical Research Council for an expenses grant from which part of the cost of this investigation was defrayed.

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## BOOKS RECEIVED

### A text-book of pathology

Edited by E. T. BELL. 5th edition. 1944. London: Henry Kimpton. Pp. 862; 448 text figs. and 4 colour plates. 45s.

As in previous editions, the editor of this standard American text-book is also the principal author. Indeed the other two contributors are responsible between them for less than 8 per cent. of the whole, namely the chapters on the mycoses and the diseases of the liver and gall-bladder by J. S. McCartney and on diseases of the heart by B. J. Clawson. The text has been brought well up to date. "Among the new topics discussed more extensively are shock, vitamin deficiencies, blast injuries, Boeck's sarcoid and several infectious diseases of interest in War Medicine". There is, however, only passing reference to the crush syndrome, on which so much valuable work has been done during the war, while the characteristic and important myocardial lesion in sarcoidosis is not mentioned. Thirty-two new figures have been added and 15 old ones deleted, and although the text is increased in amount, it has been possible to reduce the number of pages by the use of a larger type page. Selected lists of references, mainly to the American and British literature, continue to be a useful feature.

The remark to which we drew attention in our review of the third edition (this *Journal*, 1938, xlvii, 199)—that in America iron miners do not develop any clinical symptoms from the dust that they inspire—still appears (p. 124). If this is a correct statement it can only be surmised, in view of experience in this country, that either the iron stone mined is of very different composition from English hæmatite or American mining technique ensures that little or none of the dust gets into the lungs.

Proof reading has been less careful than in previous editions, which is understandable in these difficult times, but we do hope that an error referred to in our review of the second edition—the mis-spelling of Sir Robert Hutchison's name (p. 765)—will be rectified in the next edition. In passing, is it really true that neuroblastomas of the left adrenal metastasise to the skull with greater frequency than those of the right, or is this just one of those hoary fallacies which are copied from text-book to text-book?

We can only repeat, what we have said on a previous occasion, that this is an excellent students' manual. It is accurate, well written, comprehensive and yet concise, and beautifully produced.

### Leukopenia and agranulocytosis

By WILLIAM DAMESHEK. 1944. New York, London and Toronto: Humphrey Milford (Oxford University Press). Pp. vii and 78. 10s. 6d.

A compact review of the subject, almost but not quite up to date. The importance of granulotoxic drugs is stressed and the use of sulphonamides in combating general sepsis in the later stages of the disease is advocated. Necessarily there is lack of reference to the newer sulphonamides and to thiouracil. The application of penicillin is mentioned only hypothetically. The self-sufficiency of the American school of hæmatology may be judged from the fact that there is not a single reference to any British work on the subject; apart from a few references to the earlier classical German and Scandinavian papers, the whole of the bibliography is culled from American papers of the past ten years.

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